



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/385, C07K 14/00, C12N 15/11, C07K 14/705		A1	(11) International Publication Number: WO 98/33523
			(43) International Publication Date: 6 August 1998 (06.08.98)
(21) International Application Number: PCT/GB98/00325		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 2 February 1998 (02.02.98)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 9701999.6 31 January 1997 (31.01.97) GB 9714182.4 5 July 1997 (05.07.97) GB 9716620.1 7 August 1997 (07.08.97) GB 9716641.7 7 August 1997 (07.08.97) GB 9724584.9 21 November 1997 (21.11.97) GB			
(71) Applicant (for all designated States except US): BIOVATION LIMITED [GB/GB]; Auris Business Centre, 23 St. Machar Drive, Aberdeen AB24 3RY (GB).			
(72) Inventors; and (75) Inventors/Applicants (for US only): CARR, Frank, Joseph [GB/GB]; Birchlea, The Holdings, Balmedie, Aberdeen AB23 8XU (GB). CARTER, Graham [GB/GB]; Longhills Cottage, By Newmachar, Aberdeenshire AB21 7XB (GB).			
(74) Agent: BASSETT, Richard; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).			
(54) Title: VACCINATION METHODS AND MOLECULES			
(57) Abstract <p>A molecule comprising 1) a nucleic acid portion from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived and 2) a polypeptide portion, from which at least one peptide for presentation on MHC class I or class II molecules, or both, is provided. Such hybrid vaccine molecules give rise to enhanced immune responses, or to modulation of the response to the antigens which may be derived from these molecules. The combination of nucleic acid and polypeptides in the same molecule may give rise not only to a combination of MHC class I- and MHC class II-mediated immune responses but also to an enhancement of these responses compared to the responses given by either nucleic acid or polypeptide alone. The molecules may comprise Fc antibody domains which may enhance uptake of the molecules by antigen presenting cells.</p>			

Best Available Copy

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

VACCINATION METHODS AND MOLECULES

The present invention relates to molecules which can elicit desired immune responses when administered to humans or other vertebrates.

5

The mammalian organism resists the challenge of foreign antigens through its immune defence system which principally comprises two arms, namely the cellular immune system and the humoral or antibody-associated immune system. The cellular immune system is centred around the activity of T cells especially cytotoxic T lymphocytes (CTLs) and helper T cells (TH) which can recognise foreign antigens displayed on cell surfaces in a complex with major histocompatibility (MHC; known as HLA in humans) class I and class II molecules respectively. On the other hand, the humoral immune system is centred around the activity of B cells which produce antibody molecules which bind to the foreign antigen and, if the antigen is on the surface of a cell, can induce the fixation of complement or the recruitment of components of the cellular immune system to attack cells displaying the antigen (or a foreign organism).

20 The challenge of an organism by an agent which includes a proteinaceous antigen may result in a cell-mediated or a humoral or both types of immune response depending on the ability of the antigen to be taken up by antigen presenting cells (APCs) and presented by MHC class I or class II molecules. Subsequently, a cell-mediated immune response may result, in which T cells recognising a specific MHC-peptide complex are expanded and, in turn, react with other similar or identical MHC-peptide complexes on target cells. Alternatively, an antibody-mediated immune response occurs where B cells recognising a specific antigen are activated. The

enhanced response on second and subsequent exposure to an antigen is known as immunity. Immunity can be triggered by exposure of the mammal to a non-harmful form of the antigen. Such exposure may also serve to modulate the immune response to an antigen or related antigen to which the mammal has previously been exposed. The non-harmful form of the antigen, in a physiologically acceptable carrier, is a vaccine.

For specific delivery of protein-derived antigens to MHC class I or class II molecules, the protein must be processed correctly within an appropriate compartment for subsequent release and presentation of peptides on MHC class I and class II molecules. Peptide presentation on MHC class I is facilitated if the protein is processed in the cytoplasm whilst presentation on MHC class II is facilitated if the protein is processed in the endosomal compartments. Commonly, MHC class I-mediated peptide presentation is facilitated if the protein antigen is derived from within the nucleus via messenger RNA which is then translated into protein in the cytoplasm. Commonly, class II-mediated presentation is facilitated if the protein antigen is derived from outside the cells and is taken up by the cell by endocytosis or similar phenomena. As a result, exogenous protein antigens often give rise to a good MHC class II-mediated responses (especially helper T cell expansion) but poor MHC class I-mediated responses whilst antigens encoded by genes which are introduced in such a way as to enter the nucleus and undergo transcription into mRNA often give good MHC class I-mediated responses. For certain applications, for example vaccination against cancers, it appears to be important to mount both class I and class II-mediated responses; however, for exogenous antigens, this is currently difficult and there is therefore a great need for

improved vaccines which can induce effective MHC class I and class II-mediated immune responses.

5 The term polypeptide is defined as a molecule comprising at least three amino acids joined by peptide bonds. It therefore encompasses both short peptides of, for example, four to ten amino acids, and proteins, which may be of several thousand amino acids. It will be used in this manner throughout this application.

10 Previous studies have used naked DNA to give a good MHC class I-mediated immune responses (for example to influenza virus nucleoprotein as reported by Fu *et al.*, *Journal of Virology*, **71** (1997), 2715-2721) and polypeptides to give a good MHC class II-mediated immune responses (for example Hoyne *et al.*, *Clinical Immunology and Immunopathology*,
15 **80**, (1996), 523-530). Naked DNA has the disadvantage that it may give rise to genetic abnormalities. The usual uptake of DNA alone appears to be substantially non-specific and could include stem cells with long-lasting proliferative potential. This could lead to long-term persistence of DNA molecules integrated into the genes of stem cells and therefore there
20 is potential for a genetic abnormality detrimental to the organism as a result of disruption of normal gene function. In addition DNA vaccination requires immunisation with large quantities of DNA and the subsequent immune response is often dependent on the route of administration.

25 Attempts have also been made to use defined synthetic peptides, corresponding to immunodominant T-cell epitopes, to activate T-cells to specific antigens. This has relied on the efficient processing and antigen presentation by professional immune system cells to induce a

predominantly CD4⁺ helper T-cell response to the peptide presented in conjunction with MHC class II molecules. The efficiency of presentation is highly dependent on factors such as peptide concentration and peptide formulation with adjuvants, which are variable for different peptides and
5 do not always result in a specific T-cell response. In addition, exogenously added peptides will normally be taken up and processed by cells in such a way that they are presented to the immune system in association with MHC class II antigens only. Exogenously added peptide tends not to enter the processing pathway which leads to association with
10 class I MHC antigens which is essential for eliciting a CTL response.

The efficiency of antigen capture is one of the main factors in influencing the performance of antigen presenting cells (APCs; Scardino *et al.* (1994), *Immunol.* **81**, 167).

15

Fc receptors are cellular receptors for the Fc domain of immunoglobulins. The receptors are surface glycoproteins which occur on the surface of immune effector cells. There are specific receptors for different Ig isotypes. There are three classes of IgG receptors or FcR. FcRI (CD64) is
20 a high affinity receptor which binds monomeric IgG and is expressed on monocytes, macrophages and dendritic cells. There are two low affinity FcR which bind multivalent IgG immune complexes, FcRII (CD32) and FcRIII (CD16), which occur on neutrophils, eosinophils and macrophages.

FcRII is, furthermore, expressed on monocytes, B cells, basophils and
25 platelets. The FcR have common structures being composed of two (FcRII and FcRIII) or three (FcRI) extracellular Ig-like ligand-binding domains coupled to a transmembrane domain and a intracytoplasmic tail responsible for signal transduction. Binding of multiple Fcs to FcRII and

FcRIII and the binding of antigens or antigen complexes to neighbouring FcRI's leads to receptor cross-linking and activation of a signalling cascade. This then activates the FcR dependent phagocytic or endocytic response.

5

It has been demonstrated that targeting antigen to the FcRs can reduce the amount of antigen required for T-cell activation by 1000 fold (Gosselin *et al. J. Immunol.*, **149** (1992), 3477; Sallusto & Lanzavecchia *J. Exp. Med.* **179** (1994), 1109) as antigen-IgG complexes cross-link the FcR triggering
10 endocytosis or phagocytosis of the complex (Fanger *et al. J. Immunol.* **157** (1996), 541). Extracellular antigen taken up by APCs via endocytosis are mainly degraded in the endosomal compartments and this processing pathway leads to antigen presentation on MHC class II molecules and activates CD4⁺ T cells (Brander *et al. Eur. J. Immunol.* **23** (1993), 3217).
15 Induction of FcR mediated internalisation via phagocytosis can also lead to antigen presentation. It has been demonstrated that phagocytosis of exogenously added peptide by FcRs is an important mechanism for stimulating class I-restricted cytolytic T-cell responses to exogenous antigen (Falo *et al. Nature Med.* **1** (1995), 649). It is proposed that the
20 exogenous antigen enters the phagosome of the APC, from where it enters the MHC class I-restricted antigen-processing pathway (Kovacsovics-Bankowski & Rock, 1995). The mechanism of antigen uptake, by endocytosis or phagocytosis, and subsequent presentation would be dependent on the cell type bearing the FcR and the size of the
25 complex presented.

Various strategies have been employed to target antigens to FcRs. For FcRI, which is the high affinity receptor for monomeric IgG, the success

of most strategies has been limited by serum IgG which competes for binding to FcRI. An alternative strategy has been to target antigens to FcRII and FcRIII by the use of multiple Fc regions attached to the antigen.

Evidence for the efficacy of such an approach has been provided by
5 experiments performed using dimeric IgG antibodies to activate antibody-dependent cellular cytotoxicity and the complement cascade more efficiently than monomeric IgG (Kurlander & Gartrell *Blood* 62 (1983), 652; Finbloom *J. Immunol.* 136 (1986), 844; Shopes *J. Immunol.* 148 (1992), 2918) and the observation that heating of IgG causing aggregation
10 can effectively activate the complement cascade in the absence of antigen (Shopes *J. Immunol.* 148 (1992), 2918). Other strategies have employed mAbs which bind to sites on the FcRs at sites other than the ligand-binding sites (Deo *et al. Immunol. Today* 18 (1997), 127). However, whilst this triggers the complement cascade and facilitates ADCC it does
15 not necessarily permit antigen presentation.

The mechanism of DNA entry and processing stages/compartments in cells are as yet unclear.

20 The present invention is based on the surprising discovery that the combination of DNA and polypeptides in the same molecule can give rise not only to a combination of MHC class I- and MHC class II-mediated immune responses but also to an enhancement of these responses compared to the responses given by either DNA or polypeptide alone.
25 DNA-polypeptide constructs are known in the gene therapy field, for example Poncet *et al., Gene Therapy* 3 (1996), 731-738, wherein the function of the polypeptide moiety is solely to target the DNA to the required cell type, but is not known as a modulator of immune function,

and does not have the features necessary for such use, in that the polypeptide component does not contain peptides that are associated with the disease or condition being treated. A peptide or epitope is defined as being associated with a disease or condition if it is immunologically cross-reactive with an epitope whose frequency differs between the group of members of a population who are affected by the disease or condition, and the group of members of the said population who are unaffected by said disease or condition. A condition is defined as a state of health in which disease symptoms may not be evident, but in which treatment may be beneficial, for example in order to prevent the development of disease symptoms.

It has been found that induction of an immune response by DNA may be enhanced by complexes of this DNA with antibodies. Efficient vaccination may be achieved by effective targeting of DNA to professional antigen presenting cells (APCs).

The first and principal aspect of the present invention provides for such improved vaccines using hybrid polypeptide-nucleic acid molecules. These molecules comprise 1) a nucleic acid portion from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived and 2) a polypeptide portion, from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived. A Class II-mediated response may be caused primarily by the polypeptide portion, and a class I-mediated response may be caused primarily by the nucleic acid portion reaching the nucleus and thereby producing endogenous mRNA by transcription and polypeptide by translation. By the inclusion of such polypeptide and nucleic acid

domains, the hybrid polypeptide-nucleic acid molecules of the present invention may be equipped with components which provide for effective MHC class I- and class II-mediated responses. By this it is meant that the responses are modulated to an extent that can be detected using the assay systems described, or by equivalent assays known to those skilled in the art. Preferably the responses are modulated to an extent that a biologically significant difference in response to challenge by the intended antigen is achieved.

- 10 In order to select hybrid polynucleotide-nucleic acid molecules that produce the required response, the following tests may be performed. Said molecules are administered to mammals by administration routes known to those skilled in the art, preferred routes being intramuscularly, intradermally or intranasally. Granulocyte-macrophage colony stimulating factor (GM-CSF) may be co-administered when the intradermal route is used, and may enhance the immunising effect. The intradermal route would appear to give a better response than the intramuscular route for HbS antigens. An example of an appropriate immunising method for use in mice is as follows: 50 μ l samples of
- 15 hypertonic sucrose (25%w/v in phosphate buffered saline, pH 7.4; PBS) are injected bilaterally into the tibialis anterior muscles of 6 week-old anaesthetised BALB/c mice. 30 minutes later, 100 μ g samples of a molecule of the invention, or suitable doses of positive and negative comparator molecules, each in 50 μ l PBS, are injected into the same sites.
- 20 The method is applicable to other mammals with appropriate modifications. Preinjection of sucrose and anaesthesia are not required with larger animals.

MHC class I-mediated responses to HBsAg may be analysed in mice as follows. Mice are sacrificed 7 days after injection and 3×10^7 spleen cells from the mice are mixed with 10^8 peptide-pulsed and mitomycin C (Sigma)-treated spleen cells ($10 \mu\text{g/ml}$ synthetic HBsAg peptide added to $10^7/\text{ml}$ syngeneic spleen cells and incubated for 3 hours at 37°C) in 20ml Iscove's modified Dulbecco's medium (Gibco) with 10% foetal calf serum (Gibco), $50 \mu\text{M}$ β -mercaptoethanol and 20units/ml recombinant IL-2 (R and D Systems, Oxford). Spleen contains a mixture of APCs and T cells and is therefore a good concentrated source of both for mouse experiments. This is an important consideration as a mouse has only 1 ml of blood. Cells are incubated for 7 days at 37°C and then used to challenge ^{51}Cr -labeled mouse A20 (TIB208) target cells (obtained from ATCC) pulsed with the MHC class I peptide at $10 \mu\text{g/ml}$ for 1 hour at 37°C . Serial dilutions of effector cells are incubated with 2×10^3 target cells in $200 \mu\text{l}$ medium for 4 hours at 37°C . $100 \mu\text{l}$ of culture supernatants and the resuspended cell pellet are then counted. The results are analysed by ^{51}Cr release from target cells and the results are expressed as the percentage of total ^{51}Cr released by the target cells. A detectable response to the molecule of the invention is held to exist if the percentage of ^{51}Cr released is significantly increased (as assessed by statistical analysis of the data sets) where the molecule of the present invention is tested with target cells pulsed with a peptide related to a component of the molecule when compared to the percentage released where the molecule of the present invention is not added or where the target cells are pulsed with an unrelated peptide. It is preferred that the MHC class I-mediated response is such that $>25\%$ of total ^{51}Cr is released from target cells. The method is applicable to other mammals with appropriate modifications. For example, in humans, peripheral blood lymphocytes may be used as a

source of APCs and T cells (instead of spleen), although enrichment of these cell types, using known methods, is usually required to obtain clear biological results.

- 5 MHC class II-mediated responses to HBsAg may be analysed in mice as follows. The method is applicable to other mammals. Blood is collected from anaesthetised mice various times after the injections of putative antigens by retrobulbar puncture into heparinised glass pipettes. The blood is centrifuged and the serum stored at -20 °C until analysis. Anti-
10 HBsAg antibodies are measured using the AUSAB EIA test (Abbott) according to the manufacturer's instructions. For other antigens, measurement of antibody response may similarly use antigen-coated solid phases and labelled-secondary antibodies to detect antibody raised against the test antigen. A detectable response is held to exist if there is a
15 significantly increased (as assessed by statistical analysis of the data sets) level of antibody in animals that have received the putative antigen when compared with control animals that have not received said antigen. The absolute level of antibody may vary with different antigens. It is preferred that there is a five-fold increase in the level of antibody obtained with the
20 molecule of the invention as antigen over that obtained with the polypeptide portion or nucleic acid portion alone.

A biologically significant difference in response is held to exist if the difference in response measured in the above assays is equal to or greater
25 than that known from the art to equate to a biologically significant difference in response to the intended antigen in that mammal. Alternatively, for various molecules of the invention, the biological response may be measured by a range of other biological, biochemical and

phenotypic end-points such as delayed-type hypersensitivity (DTH) skin reactions to measure T cell responses, interleukin-2 production to measure TH responses and reversal of disease state (for example, shrinkage of a tumour in cancer).

5

In addition a portion of the molecule may comprise a recognition domain.

This may anchor the vaccine molecule to a cell surface antigen or receptor. These various domains may be juxtaposed in a variety of configurations.

10

The invention further provides a molecule comprising 1) a nucleic acid portion from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived and 2) a polypeptide portion comprising a recognition domain capable of targeting the molecule to an
15 antigen presenting cell (APC) wherein the polypeptide portion does not comprise a specific antigen binding site.

For example, the molecule may comprise a binding portion ("recognition domain") capable of binding to one or more FcR molecules and an
20 effector portion comprising nucleic acid which is capable of exerting a biological effect, wherein binding of the binding portion induces internalisation of the chimaeric molecule such that the biological effect of the effector portion is enhanced (or facilitated).

25 Preferably the polypeptide portion comprises an immunoglobulin molecule (for example an antibody) or an effective portion thereof which does not comprise a specific antigen binding site. The term "effective portion" is intended to denote a portion of an immunoglobulin molecule

which is able to bind to an FcR molecule with sufficient affinity to be useful in the present invention, for example the Fc region or portion thereof.

5

By a specific antigen binding site (ie what is disclaimed) is included an immunoglobulin variable region, Fv, Fab or recombinant single-chain Fv fragment or other immunoglobulin-derived, for example antibody-derived, fragment which retains a specific binding site which may be prepared by techniques as described in Winter & Milstein *Nature* **349**, 293-299 and other references listed below. An immunoglobulin constant region is not included. The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parent antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

20

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**,

5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). Fragmentation of intact immunoglobulins to produce F(ab')₂ fragments is disclosed by Harwood *et al* (1985) *Eur J Cancer Clin Oncol* **21**, 1515-1522.

5

By Fc region is meant the Fc region of an antibody, preferably a human antibody, for example as defined in Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855. Thus an "effective portion" of an immunoglobulin molecule may comprise a polypeptide with at least 50%,
10 80%, more preferably 85%, still more preferably 90% and still more preferably 95% or 98% homology with at least part of a sequence defined as a human antibody constant region in Morrison *et al* (1984)) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855. The effective portion may have the above-defined homology with all or substantially all of the defined constant
15 region sequence or with one or more contiguous sequences that comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the defined constant region sequence.

The effector portion of the chimaeric molecule preferably is or comprises
20 DNA or another nucleic acid molecule or a combined nucleic acid-polypeptide molecule comprising nucleic acid conjugated to one or more polypeptides. The effector component comprises a nucleic acid portion as defined above and an optional polypeptide portion, as defined in the first aspect of the invention. As for the first aspect of the invention, a class II-
25 mediated response may be caused primarily by the polypeptide portion, and a class I-mediated response may be caused primarily by the nucleic acid portion reaching the nucleus and thereby producing endogenous

mRNA and hence polypeptide. In addition, proteins derived from (ie encoded by) the nucleic acid portion may be secreted.

5 A molecule of the present invention may comprise one or more polypeptides attached to a single nucleic acid molecule. The polypeptide (or polypeptides) may be an antibody and may be attached to the nucleic acid via a natural specificity of the antibody for a nucleic acid or via chemical conjugation or by ionic interaction.

10 Molecules of the present invention may comprise anti-DNA antibodies (especially those which bind to double-stranded DNA) attached to the DNA, and/or antibodies with other specificities to which the DNA is attached, for example chemically. Other variant chimaeric molecules of the present invention may comprise an antibody with specificity for
15 derivatised nucleotides contained in the nucleic acid. For example, one or more synthetic oligonucleotides containing one or more derivatised nucleotides may be introduced into the nucleic acid molecule by annealing to complementary sequences in the nucleic acid molecule either by total or partial denaturation of double-stranded DNA molecules or use of single-
20 stranded DNA (or RNA). Where required, single-stranded DNA (or RNA) with the derivatised annealed synthetic oligonucleotide can be rendered double-stranded by addition of DNA (or RNA) polymerase and deoxynucleotides to effect primer extension.

25 Other variant chimaeric molecules of the present invention may comprise an antibody with specificity for a derivatised nucleotide or nucleotides introduced into the nucleic acid by means other than with synthetic oligonucleotides, such as by using specific restriction endonucleases to display single-stranded ends in DNA which can then be filled in to include

one or more derivatised nucleotides. This DNA may, if required, then be recircularised using a DNA ligase. Still further variant chimaeric molecules of the present invention may comprise an antibody with specificity for a non-nucleic acid molecule associated with the nucleic acid, such as a protein molecule or protein molecules conjugated to the DNA, whereby one or more antibody molecules bind to the protein or proteins. Such proteins may include proteins which naturally bind to certain sequences in the DNA. For example, the DNA may include a bacterial *lac* operator sequence to which 4 molecules of the *lac* repressor may bind. Antibodies specific for the *lac* repressor may then bind to the *lac* repressor, resulting in a chimaeric DNA-antibody molecule. Still other chimaeric molecules of the present invention may comprise an antibody or antibodies with specificity for non-nucleic acid molecules such as intercalators complexed with DNA and dyes complexed with DNA or RNA whereby, in total, one or more antibody molecules bind to these molecules.

It will be understood by those skilled in the art that chimaeric molecules of the present invention may also be generated by other routes or methods.

20

The present invention may use antibodies or the Fc region of an antibody singly or in multiple copies to target the antigens to FcRs on APCs, leading to cross-linking of FcRs, internalisation and subsequent expression in conjunction with MHC class I and/or class II molecules. Advantages of this aspect of the invention for the activation or tolerance of T-cells may be several fold. By targeting nucleic acids to FcRs and effecting subsequent internalisation by FcRs, the efficiency of subsequent antigen presentation by APCs may be increased. Several different nucleic acid

sequences may be contained in a single internalised construct thus providing for a choice of peptides for presentation by the appropriate MHC haplotype in the individual immunised.

- 5 Employing Fc receptor mediated delivery of nucleic acid may enable efficient delivery of the effector molecule to APCs as an alternative to the less specific pinocytosis by these cells or the less efficient carrier-mediated uptake e.g. using transferrin which necessitates the use of high concentrations of transferrin constructs to sensitise target cells (Brander *et*
10 *al. Eur. J. Immunol.* 23 (1993), 3217).

It will be understood by those skilled in the art that the binding portion (recognition portion) of a molecule of the present invention may comprise a single antibody or Fc component with the ability to bind to a single Fc
15 receptor or it may comprise more than one antibody and/or Fc component with the ability to bind to adjacent (or neighbouring) Fc receptors. The multiple (ie more than one) antibodies and/or Fc components may themselves be adjacent or neighbouring. Whilst neighbouring antibody or Fc components may arise simply by virtue of the position at which they
20 bind to the nucleic acid of the molecule of the invention, it will be understood that the antibodies or Fc components may also be cross-linked in order to achieve the same result of binding neighbouring Fc receptors. It will be understood that cross-linking of antibody or Fc components should be such that binding of the Fc region to FcRs is not impaired. The
25 protein domains may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al.*, (*Anal. Biochem* 100, (1979), 108). For example, these domains could be linked chemically using a cross-linking agent such as the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-

succinimidyl-3-(2-pyridyldithio)propionate (SPDP) which react with thiol groups. Such cross-linking may be facilitated by the introduction of specific amino acids into one or both of the domains, especially free cysteine residues or free lysine residues which do not impair the binding
5 function of the Fc region to the FcR.

The targeting antibody may be of the IgG class and most preferably human, alternatively humanised, for example as described in Example 10.

This may reduce the immunogenicity of the antibody. The isotype of the
10 antibody may be altered to target specific classes of FcR i. e. FcRII has highest affinity for IgG3 and FcRIII has highest affinity for IgG1 (Deo. *et al.*, *Immunol Today*, 18 (1997), 127). The antibody may be a monoclonal antibody. The antibody may be attached to the effector molecule either singly or in multiple copies such that the Fc regions may be capable of
15 binding FcR's on APCs leading to internalisation of the antibody and effector molecule.

It will be understood by those skilled in the art that the immunogenicity of the constructs could be further increased by co-administration of
20 appropriate cytokines. It will be appreciated that these may be considered to act as immunological adjuvants. For instance it has been demonstrated that FcRII expression on cultured dendritic cells is substantially increased in the presence of GM-CSF and IL-4 but inhibited in the presence of TNF- α (Sallusto & Lanzavecchia, *J. Exp. Med.*, 179, (1994), 1109).

25

Molecules of the present invention may have several advantages over molecules in the prior art. The specific targeting of FcR may limit the uptake of DNA molecules to APCs, in contrast to the usual uptake of

DNA alone which appears to be less specific and may include stem cells with long-lasting proliferative potential. By concentrating the uptake of DNA on APCs which will subsequently terminally differentiate, the present invention may limit the long-term persistence of DNA molecules integrated into the genes of stem cells and therefore limit the potential for a genetic abnormality detrimental to the organism as a result of disruption of normal gene function. A second advantage over naked DNA vaccination may be that targeting uptake to APCs may reduce the amount of DNA required to elicit an effective immune response as it is well documented that efficiency of antigen capture is one of the main factors in influencing the performance of APCs (Scardino *et al. Immunol.*, 81 (1994), 167).

A peptide is defined as a molecule comprising at least two amino acids joined by a peptide bond. The said polypeptide portion of the molecule of the invention preferably comprises one or more fragments of a natural protein comprising one or more peptides which are suitable for loading onto MHC molecules. Preferably, a said peptide has between four and fifty amino acids, more preferably six to thirty-five amino acids, and most preferably eight to twenty amino acids. The suitability of a said peptide for presentation on MHC class I or Class II molecules can be assessed by searching for linear amino acid motifs which are known peptides which bind to MHC molecules (see, for example, Altuvia *et al. Molecular Immunology*, 31 (1994), 1-19). However, the flanking sequences of peptides within a protein also influence the processing of a peptide for subsequent presentation by MHC molecules, so this method may not be fully predictive.

Examples of suitable antigens or peptide epitopes include, but are not limited to: known antigenic determinants, super-antigens (by which is meant antigens which are capable of eliciting a dominant immune response in the presence of other antigens, for example hsp60 heat shock proteins), epitopes which are known to produce a strong immune response, and epitopes that are associated with a particular disease or condition. Preferably, at least one of the peptides which may be derived from the polypeptide portion, may be associated with a disease or condition. It will be appreciated that a peptide may be derived from the polypeptide portion by processing by an antigen presenting cell, as described above.

It will be appreciated that in a chimaeric molecule of the second aspect of the invention, these antigens may be encoded by the nucleic acid or may optionally be derived from the polypeptide attached to the nucleic acid, or both.

At least one of the peptides that may be derived from the polypeptide portion may be immunologically cross-reactive with a peptide derivable from a polypeptide found in a pathogenic organism, or from a protein found in leukaemia, prostate cancer, breast cancer, lung cancer, liver cancer or bowel cancer cells. Such polypeptides may be, but are not limited to, intact proteins. Examples of polypeptides that may contain suitable peptides include, but are not limited to surface or internal polypeptides of: a pathogenic organism (including all stages of the organism's life cycle), a virus particle, a cell infected with a virus, a bacterium, a cell harbouring an intracellular pathogen, or a transformed or malignant cell. Examples of such polypeptides include viral antigens such as Hepatitis B surface antigen (HBsAg; Schirmbeck *et al.*, *J.*

virology, 68 (1994), 1418), bacterial antigens such as tetanus toxin (Panina-Bordignon *et al.*, *Eur. J. Immunol.*, 19 (1989), 2237), parasitic antigens (Jaso-Friedmann *et al.*, *Cellular Immunology* 176 (1997), 93), and tumour antigens such as tyrosinase (Brichard *et al.*, *J. Exp. Med.*, 178 (1993), 489). The virus may be Hepatitis B. More particularly, the polypeptide may be the surface antigen HBsAg.

In particular the tumour antigen may be CD55 or carcinoembryonic antigen (CEA), both antigens associated with cancer cells, particularly colorectal cancer cells, and well known to those in the art. Known CEA epitopes include those mentioned in Tsang, KY *et al* (1995) *J Natl Cancer Inst* 87, 982-990. Peptide epitopes may be selected from those predicted to be presented by MHC Class I or Class II molecules and may in particular be peptides that are present in CEA but not in related proteins, for example, other members of the "gene family" to which CEA belongs. The peptides derived from CEA and CD55 and encoded by the constructs of Example 10, for example the "CEA-mini-gene" are particularly preferred.

Further examples include antigens involved in diseases or conditions in which the aetiology of the disease or condition involves allergy. Examples include, but are not limited to, atopic dermatitis, allergic rhinitis, allergic conjunctivitis, atopic asthma or eczema. Such an antigen may be a peptide which is immunologically cross-reactive with a peptide derivable from a polypeptide found in pollen, animal dander, house dust mites or washing detergent enzymes. Such polypeptides may be, but are not limited to, intact proteins. Examples include, but are not limited to, grass pollen antigens (Cao *et al.*, *Immunology* 90 (1997), 46) and house

dust mite antigens (Harris *et al.*, *International Immunology* 9 (1997), 273-280).

Still further examples include antigens associated with an autoimmune
5 disease or condition. Examples of such diseases or conditions include, but
are not limited to Grave's disease, multiple sclerosis, systemic lupus
erythematosus (SLE), diabetes mellitus, Kawasaki's disease or rheumatoid
arthritis. Such an antigen may be a peptide which is immunologically
cross-reactive with a peptide derivable from a polypeptide found in the
10 thyroid, myelin sheath cells, nucleoproteins, pancreatic islet cells,
mucocutaneous lymph nodes, bones or synovial joints. Such polypeptides
may be, but are not limited to, intact proteins. Examples of suitable
polypeptides may be found in Molnar *et al.*, (1995) *J. Endocrin. Invest.*
18, 408-414 (Grave's disease), Utz *et al.*, (1994) *PNAS USA* 91, 5567-5571
15 (multiple sclerosis), Amasaki *et al.*, (1995) *Clin. Exp. Immunol.* 99, 245-
250 (SLE), Richter *et al.*, (1995) *Eur. J. Immunol.* 25, 1703-1712 (diabetes
mellitus), Vagliasindi *et al.*, (1997) *Acta Paediatrica, Int. J. Paediatr.* 86,
317-318 (Kawasaki's disease), Wagatsuma *et al.*, (1996) *Mol. Immunol.*
33, 1171-1176 (rheumatoid arthritis).

20

In all examples, the polypeptide portion may comprise an anti-idiotypic
immunoglobulin or effective portion thereof which can bind to
immunoglobulins capable of binding to an antigen or polypeptide
discussed above. By this is meant an immunoglobulin which has an
25 epitope that is immunologically cross-reactive with the antigen or
polypeptide. The immunoglobulin may comprise peptide sequences of the
antigen or polypeptide, which may be inserted in the CDR regions of the
immunoglobulin molecule.

For example, the immunoglobulin may comprise peptide sequences found in CEA or CD55, for example as described in Example 10. A particularly preferred construct, as described in Example 10, is that in which the following peptides are inserted at the following positions of the immunoglobulin:

- amino acids 629 to 645 of CEA (₆₂₉CEA₆₄₅) inserted into Vh CDR2
- amino acids 386 to 397 of CEA (₃₈₆CEA₃₉₇) inserted into Vh CDR3
- amino acids 345 to 354 of CEA (₃₄₅CEA₃₅₆) inserted into Vk CDR1
- 10 amino acids 571 to 579 of CEA (₅₇₁CEA₅₇₉) inserted into Vk CDR3

Alternatively an alternative segment of CEA sequence (specifically ₂₈CEA₃₆) may be inserted into the heavy chain variable region (CDRH3), for example of mAb 708, also as described in Example 10.

15

Amino acids 182 to 201 of CD55 (₁₈₂ CD55₂₀₁) may also be inserted, for example as shown in Example 10.

The following references refer to CEA peptides: Tsang KY *et al* (1995) *J Natl Cancer Inst* 87, 982-990 and Durrant LG *et al* (1992) *Int J Cancer* 20 50, 811-816.

The polypeptide domain (which refers also to the optional polypeptide component of the effector portion of the chimaeric molecule of the second aspect of the invention) is preferably between four and four thousand 25 amino acids in length. If the polypeptide portion consists of a peptide which is suitable for loading onto MHC molecules, it preferably has between four and fifty amino acids, more preferably six to thirty-five

amino acids, and most preferably eight to twenty amino acids. If the polypeptide portion comprises a protein or an array of individual peptides, it will preferably be between fifty and one thousand amino-acids in length, most preferably between two hundred and six hundred amino-acids in length.

The polypeptide portion of the molecule of the invention may be produced using recombinant DNA techniques. Thus, the DNA encoding the polypeptide portion of the molecule of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide portion of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the
5 desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression
10 vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

15 Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

20

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

25

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an

appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

- 5 A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

10

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

- 15 A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

- 20 An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

- 25 Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers

HIS3, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps)

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

- 5 A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

10 In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

- 15 The nucleic acid portion, from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived, preferably encodes one or more fragments of a natural protein comprising peptides which are suitable for loading onto MHC class I or class II molecules. It may encode a complete or substantially complete natural protein.

20 Preferably, the nucleic acid is double-stranded DNA, less preferably RNA. The DNA is preferably autonomously replicating, most preferably a plasmid, less preferably of viral origin. The DNA may be capable of insertion into the host DNA. Expression of the polypeptide may be constitutive or capable of being regulated; preferably it is constitutive.

- 25 Expression may be possible in all cell types, or may be restricted to particular cell types; preferably it is possible in all cell types. Cell types to which expression may be restricted are preferably Antigen presenting cells, for example dendritic cells. This may be achieved by the use of

promoter regions such as those from the MHC class II genes, from the mannose receptor genes, from Fc receptor genes and from CD63 genes. Constitutive expression may be achieved by use of a viral promoter, examples including but not limited to the cytomegalovirus, CMV, and
5 respiratory syncytial virus, RSV, promoters. Examples of plasmids include, but are not limited to: pRc/RSV, pRc/CMV (Invitrogen), pMAMneoBlue (Clontech) and pRK.

Examples and preferred options of polypeptides and peptides that may be
10 encoded by the nucleic acid portion of the molecule of the invention, and the peptides that may be derived from them for loading onto MHC class I or class II molecules, include the examples and preferred options given for peptides that may be derived from the polypeptide domain of the molecule of the invention for loading onto MHC class I or class II molecules.

15

A particular example of a molecule of the invention is characterised in that at least one of the peptides which may be derived from the nucleic acid portion has a region which is substantially identical to a region of at least one of the peptides which may be derived from the polypeptide portion.
20 Substantially identical is defined in this context as having the same epitopes.

It is particularly preferred that at least one peptide which may be derived from the nucleic acid and at least one peptide which may be derived from
25 the polypeptide (for example, immunoglobulin) portions of the molecule of the invention may have regions which are substantially identical to each other and, even more preferably are substantially identical to a region found in CEA. It is further preferred that there are two or three such

regions in a molecule of the invention. It is still further preferred that the regions are as described above and in Example 10.

It will be further understood by those skilled in the art that the nucleic acid
5 portion of the hybrid polypeptide-nucleic acid molecules may encode
molecules such as cytokines which might activate the target APCs to
enhance the immune response. An example is GM-CSF. Other examples
include, but are not limited to, other lymphokines, cytokines or factors
which have a direct biological effect on the APC itself or on cells in
10 proximity to the APC. Examples of these factors are interleukin-2,
interferon gamma, tetanus toxoid and antibodies which activate APCs, for
example anti-IgD antibody which cross-links IgD on pre-B cells and
causes activation.

15 Construction of the nucleic acid portion described can be achieved by the
use of recombinant DNA techniques well known to those in the art, and as
described above. The DNA construct of the invention may then be
purified from the host cell using well known methods.

20 For example, plasmid vector DNA can be prepared on a large scale from
cleaved lysates by banding in a CsCl gradient according to the methods of
Clewell & Helinski (1970) *Biochemistry* 9, 4428-4440 and Clewell (1972)
J. Bacteriol. 110, 667-676. Plasmid DNA extracted in this way can be
freed from CsCl by dialyse against sterile, pyrogen-free buffer through
25 Visking tubing or by size-exclusion chromatography.

Alternatively, plasmid DNA may be purified from cleared lysates using ion-exchange chromatography, for example those supplied by Qiagen. Hydroxyapatite column chromatography may also be used.

- 5 The optional recognition domain of the hybrid polypeptide-nucleic acid molecule is preferably a polypeptide, but optionally RNA.

In the former case, the polypeptide portion comprises an immunoglobulin molecule or portion thereof. It may comprise a complete immunoglobulin, preferably an antibody, more preferably of the IgG class, and most preferably human, or humanised as described above. The antibody can be a monoclonal antibody. A complete antibody comprising two heavy and two light chains with variable and constant regions may be used. In this case, a DNA molecule may be attached to each polypeptide chain or just to one chain. Preferably it is attached to one chain only, especially the heavy chain. A smaller immunoglobulin fragment, especially a variable region comprising a single heavy chain derived variable region (V_H) and a single light chain derived variable region (V_L) either in the form of an Fv, Fab or recombinant single-chain Fv fragment may be used. The recognition domain preferably binds to a cell surface antigen, preferably on an antigen-presenting cell (APC), most preferably where this antigen internalises either spontaneously or as a result of antibody binding. Examples include, but are not limited to, the FcRI receptor on macrophages, surface immunoglobulin molecules on B cells, or mannose receptors on dendritic cells.

Recognition may also be enhanced when a complete antibody is used for the third domain, in that for specific heavy chain constant region isotypes

such as human IgG1, the constant region (Fc) will contain sequences which can bind to other antigens on APCs either directly to the Fc receptors common on APCs or indirectly via complement binding to the antibody which can then bind to complement receptors on the APCs. For
5 this reason, use of a complete antibody is the preferred embodiment. The recognition portion may consist of the above constant region sequences without V_H and V_L regions.

The recognition domain may be the Fc region of an antibody that binds
10 specifically to the nucleic acid of the molecule of the invention or to a protein bound to the nucleic acid, as described earlier. Thus a further aspect of the invention is a molecule as described above wherein the nucleic acid portion is bound to the polypeptide portion by means including a specific interaction between 1) a part of the polypeptide
15 portion that comprises an immunoglobulin domain that is capable of interacting specifically with the nucleic acid and 2) the nucleic acid. A still further aspect is a molecule of the invention as described above wherein the nucleic acid portion is bound to the polypeptide portion by means including a specific interaction between 1) a part of the polypeptide
20 portion that comprises an immunoglobulin domain that is capable of interacting specifically with a polypeptide that is bound to the nucleic acid 2) the polypeptide that is bound to the nucleic acid.

It will be appreciated that the recognition domain may comprise more than
25 one molecule, for example, more than one polypeptide molecule. Thus a molecule of the invention may comprise a nucleic acid molecule to which are attached one or more polypeptide molecules. For example, more than one Fc region may be attached to a nucleic acid molecule.

The optional third type of domain of the hybrid protein-nucleic acid vaccine molecule may comprise RNA to bind the vaccine molecule to a cell surface antigen, as RNA has been shown to be capable of efficiently
5 binding to certain antigens (Tuerk & Gold, *Science* **249** (1990), 505).

Thus, an RNA component of the hybrid nucleic acid-protein vaccine molecule might include both RNA sequences for binding to an antigen and RNA sequences coding for an antigenic protein or peptides.

10

The first protein and second DNA domains of the hybrid protein-DNA vaccine molecules can be linked chemically using materials such as benzoquinone, as described by Poncet *et al.*, (*Gene Therapy*, **3** (1996), 731-738), which is incorporated herein by reference. An representative
15 protocol is given: to 2mg/ml peptide in phosphate buffered saline (pH 7.4); PBS was added 1/10th final volume of *p*-benzoquinone (Sigma, B-1266) at 30mg/ml in 100% ethanol and 1/10th final volume of 1M potassium phosphate buffer, pH 6. The mixture was incubated for 1 hour at room temperature in the dark and then passed through a Sephadex
20 G50M column (Pharmacia, Milton Keynes) which had been presaturated in 1% BSA in 0.15M NaCl. Elutions were monitored at 410nm and the initial peak containing the activated peptide was collected. To this was added equimolar HBsAg DNA and 1/10th final volume of 1M sodium carbonate/bicarbonate buffer pH 8.7 was added. The mixture was
25 incubated with slow rotation at 4 °C in the dark for 48 hours. To remove unconjugated peptides, the sample was then passed through a Bio-Gel HTP column (DNA grade, Biorad, Hemel Hempstead) and eluted with PBS according to the manufacturer's instructions. The resultant sample

was then run on a 0.8% agarose gel and the peptide-DNA conjugate was purified using an Elutip cartridge (Schleicher and Schuell, Dassel, Germany) according to the manufacturer's instructions, whereby molten gel slices containing DNA are applied to the Elutip column such that the matrix binds the DNA which is subsequently washed off in high salt buffer. The purified conjugate was then passed through a Sepharose 6HR FPLC column run in PBS and the leading fraction containing the conjugate was collected and stored at -20 °C prior to experimentation.

Alternatively, conjugation may be facilitated by the introduction of specific amino acids into the polypeptide portion and specific derivatised nucleotides into the nucleic acid portion. For example, one or more free cysteines introduced into the polypeptide by recombinant DNA manipulations well known to those skilled in the art, as described above, could be cross-linked to one or more free primary amino groups introduced into the nucleic acid portion by introduction of modified nucleotides either at the termini of the nucleic acid, for example by direct chemical modification of the DNA, such as conversion of the 5'-phosphate group into a carbodiimide derivative by treatment with ethylene diamine-carbodiimide (EDC), or internally using nick translation, PCR or transcription reactions to introduce modified nucleotides, for example biotinylated nucleotides available from several suppliers including Life Technologies. Where additional third domains are included, combinations of these linkage methods can be used and the additional protein domains can be attached to each other or directly to the nucleic acid domain. The protein domains can be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al.*, (*Anal Biochem* 100, (1979), 108). For example, these domains

could be linked chemically using a cross-linking agent such as the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) which react with thiol groups. Such cross-linking may be facilitated by the introduction of specific amino acids
5 into one or both of the domains, especially free cysteine residues (i.e. not involved in disulphide interactions) or free lysine residues (i.e. not involved in critical interactions via the primary amine group). This is achieved by recombinant DNA manipulations well known to those skilled in the art, as described above.

10

Preferably, the first and optional third domains of the hybrid vaccine molecule may be produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two portions of the polypeptide portion of the compound of
15 the invention either adjacent to one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the compound. Conceivably, the two portions of the compound may overlap wholly or partly. The DNA is then expressed in a suitable host to produce a polypeptide comprising the polypeptide portion of the
20 compound of the invention as described above.

The first and second domains of the hybrid protein-DNA vaccine molecules of the present invention are preferably arranged adjacent to each other, but can also be arranged in discontinuous segments which
25 give functional immune responses; for example, the hybrid vaccine molecule may have N- and C- terminal protein segments adjacent to one or more internal DNA molecules. This is achieved by sequential linking

of the nucleic acid and polypeptide portions, by the use of methods described above.

The nucleic acid portion of the molecule of the invention may encode one
5 or more individual peptides, or may encode a polypeptide that may correspond to a natural protein. Where the nucleic acid portion encodes an individual peptide, it will preferably be between 50 and 5000 nucleotides long, more preferably between 100 and 3000 nucleotides long. Where the nucleic acid portion encodes a protein or an array of
10 individual peptides, it will preferably be between 100 and 25000 nucleotides long, more preferably between 1000 and 10000 nucleotides long, and most preferably between 2000 and 5000 nucleotides long.

Molecules of the present invention may have several advantages over
15 molecules in the prior art. Firstly, the hybrid protein-nucleic acid vaccine molecules, as exemplified by protein-DNA molecules, provide components which may result in both efficient MHC class II-mediated peptide presentation (expected to be via the protein component) and efficient MHC class I-mediated peptide presentation (expected to be via
20 the DNA component) whereby the efficiency of the resultant cellular and humoral immune responses can be higher than those elicited by the individual components alone. Therefore, a single type of molecule may induce effective MHC class I and II-mediated immune responses such as CTL and T helper responses. A second potential advantage is that the
25 protein component of hybrid protein-DNA vaccine molecules can potentially limit the uptake of these vaccine molecules to APCs, in contrast to the usual uptake of DNA alone which appears to be less specific and can include stem cells with long-lasting proliferative

potential. By concentrating the uptake of molecules on APCs which will subsequently terminally differentiate, the hybrid protein-DNA vaccine molecules of the present invention can potentially limit the long-term persistence of DNA molecules integrated into the genes of stem cells and therefore limit the potential for a genetic abnormality detrimental to the organism as a result of disruption of normal gene function. Uptake by APCs may be facilitated by the presence of the optional third recognition domain. Intradermal administration may also facilitate uptake by APCs in draining lymph nodes, although uptake by skin cells may also be involved (Raz *et al*, *Proc Natl Acad Sci (USA)* 91 (1994), 9519). A third potential advantage especially over naked DNA vaccination is that immune responses may be focused onto specific MHC class I or class II-restricted peptides through representation of these peptides in both the protein and DNA components of the molecule. This may be important for reducing immune responses directed to other MHC presented peptides which could be derived from the protein encoded by the DNA. These other responses may not influence the disease or may even cause toxic side effects and thus may limit the effectiveness of the vaccination.

It will be understood that the hybrid polypeptide-nucleic acid vaccine molecules of the present invention have a range of medical and veterinary applications, including but not limited to vaccination against allergies and diseases caused by pathogenic organisms, induction of an immune response to cancers, and treatment of autoimmune diseases and conditions.

Examples of diseases or conditions, whose aetiology involves allergy, that can be treated include but are not limited to atopic dermatitis, allergic rhinitis, allergic conjunctivitis, atopic asthma and eczema. Examples of antigens include polypeptide components of pollen, animal dander, house

dust mites and washing detergent enzymes. Examples of cancers include, but are not limited to: leukaemia, prostate cancer, breast cancer, lung, liver, bowel cancer. Examples of cancer antigens include CD55, CEA, MART-1, gp100, tyrosinase and gp75 (Kawakami *et al.*, *J. Exp. Med.*, **180** 5 (1994), 347). Examples of autoimmune diseases include, but are not limited to, multiple sclerosis, diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, Kawasaki's disease and Grave's disease. Examples of antigens include those found in myelin sheath cells, pancreatic islet cells, nucleoproteins, bones or synovial joints, 10 mucocutaneous lymph nodes, or the thyroid. Examples of antigens include, but are not limited to those described in Molnar *et al.*, (1995) *J. Endocrin. Invest.* **18**, 408-414 (Grave's disease), Utz *et al.*, (1994) *PNAS USA* **91**, 5567-5571 (multiple sclerosis), Amasaki *et al.*, (1995) *Clin. Exp. Immunol.* **99**, 245-250 (SLE), Richter *et al.*, (1995) *Eur. J. Immunol.* **25**, 15 1703-1712 (diabetes mellitus), Vagliasindi *et al.*, (1997) *Acta Paediatrica, Int. J. Paediatr.* **86**, 317-318 (Kawasaki's disease), Wagatsuma *et al.*, (1996) *Mol. Immunol.* **33**, 1171-1176 (rheumatoid arthritis).

It will also be understood that the antigenic components of the hybrid 20 polypeptide-nucleic acid vaccine molecules will be selected for the particular application and may include components for the generation of peptides presented by MHC class I only, MHC class II only or by both MHC class I and II. Antigens will be selected from those associated with a particular disease or condition, as described above. The basis for 25 selection of peptides for MHC class I or MHC Class II presentation will be by, for example, analysing peptides for linear amino acid motifs which are known to be present in peptides which bind to MHC molecules or by selecting peptides which are known to elicit specific MHC class I or MHC

- class II-mediated immune responses. Complexes of MHC and peptide are recognised by T cell receptors (TCR) and this interaction of MHC-peptide and TCR may either up-regulate or down-regulate the T cell or may block the usual T cell response. The up-regulation of subpopulations of T cells
- 5 may lead to tolerance of a T cell response to a particular antigen or may even deviate the normal T cell response towards a particular antigen thus reducing the response of the T cell population to another antigen. For example, a TH2-type allergenic response to a particular antigen may be reduced by strong induction of a TH1 response by a hybrid vaccine
- 10 antibody vaccine molecule with the resultant production of TH2-inhibiting cytokines. In autoimmune diseases where TH1-type responses might be more important as a result of self-antigen presentation, the presentation of peptides which are similar, but not identical, to those originating from self antigens might serve to block the usual T cell response to the self antigen.
- 15 By similar peptides, what is meant is partially immunologically cross-reacting, ie sharing some but not all epitopes. Alternatively, T cell hyperstimulation by effective MHC-mediated presentation of the self peptide might produce tolerance to the self antigen.
- 20 It will also be understood that, for any particular application, one or a combination of hybrid polypeptide-nucleic acid vaccine molecules might be used for that application, for example a combination of two molecules each comprising or encoding different antigens which, when combined, result in the desired effect. It will also be understood that an individual
- 25 polypeptide-nucleic acid molecule could provide, from the same molecule, peptides derived from different antigens for presentation by MHC class I and MHC class II such that multiple beneficial effects could be achieved, for example vaccination against more than one type of infectious disease

or vaccination against various epitopes of a rapidly mutating virus or cancer antigen. This would be achieved by including peptides or coding sequences from different infectious agents, or by including multiple peptides or coding sequences differing in particular mutations, respectively.

A further aspect of the invention provides a method of vaccinating a mammal, the method comprising administration to the organism of the components described above. This can be achieved by administration in any suitable way, usually parenterally, for example intravenously, intraperitoneally, or intravesically, in standard sterile, non-pyrogenic formulations of diluents and carriers. Effective dosage ranges will be between 1 pmole and 1 mmole, preferably between 100 pmoles and 10 μ moles, and most preferably between 10 nmoles and 1 μ mole. The immune responses achieved by hybrid protein-DNA vaccine molecules of the present invention may be enhanced by mixing with immunological adjuvants which may enhance the immunogenicity of these molecules. Examples include Freund's complete and incomplete adjuvants. Activating co-stimulatory factors and cytokines such as, but not limited to, GM-CSF may be co-administered in order to activate APCs, especially dendritic cells.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the

active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral
5 administration may be presented as discrete units such as capsules, cachets
or tablets, each containing a predetermined amount of the active
ingredient; as a powder or granules; as a solution or a suspension in an
aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid
emulsion or a water-in-oil liquid emulsion. The active ingredient may also
10 be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or
more accessory ingredients. Compressed tablets may be prepared by
compressing in a suitable machine the active ingredient in a free-flowing
15 form such as a powder or granules, optionally mixed with a binder (eg
povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent,
preservative, disintegrant (eg sodium starch glycolate, cross-linked
povidone, cross-linked sodium carboxymethyl cellulose), surface-active or
dispersing agent. Moulded tablets may be made by moulding in a suitable
20 machine a mixture of the powdered compound moistened with an inert
liquid diluent. The tablets may optionally be coated or scored and may be
formulated so as to provide slow or controlled release of the active
ingredient therein using, for example, hydroxypropylmethylcellulose in
varying proportions to provide desired release profile.

25

Formulations suitable for topical administration in the mouth include
lozenges comprising the active ingredient in a flavoured basis, usually
sucrose and acacia or tragacanth; pastilles comprising the active ingredient

in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The molecules of the invention could be introduced *ex vivo* by addition to cells outside the body followed by injection of these cells into the

organism. In particular, these molecules could be used with isolated APCs, especially dendritic cells, which would be activated *ex vivo* before re-injection, using, for example, antibodies against CD34 to isolate the dendritic cells and GM-CSF/TNF α during culture (Siena *et al.*, *Exp. Haematol.* 23 (1995) 1465-1473). Dendritic cells may be isolated by methods such as those using magnetic beads, as described in the publication "MACS and more" (vol 1, no 1 (1997), Miltenyi Biotech, Bergisch Gladbach, Germany), and cultured in cytokines such as GM-CSF, TNF α and IL-4 depending on the source of the dendritic cells. The vaccine molecules would be added to the cultures, typically in the dose range of 1 μ M to 1 mM, and the culture continued for a period, for example 1 day, in order for dendritic cells to take up and process the vaccine. The cells would then typically be injected back into the patient. Optionally, the cultures could be washed prior to injection in order to remove excess vaccine molecules. One advantage of this method is that lower quantities of the molecules of the invention are required than for *in vivo* treatment. A second advantage of this method is that degradation of the molecules of the invention prior to being taken up by cells is reduced. The present invention is illustrated but not limited by the following examples;

Example 1:

DNA encoding Hepatitis B surface antigen (HBsAg) was conjugated to a equimolar mixture of the synthetic HBsAg peptide AVYYCTRGYHGSSLY which is an MHC class II-restricted HBsAg peptide (Rajadhyaksha and Thanavala, *Proc Natl Acad Sci (USA)*, 92 (1995), 1575-1579) and the synthetic MHC class I-restricted peptide CRILTAIPQSLDSWWTSLANFGG (Schirmbeck *et al.*, *J Virology*, 68

(1994), 1418-1425). For DNA encoding HBsAg, the vector pCMV-HBs was re-created as described by Davis *et al.*, *Human Molecular Genetics*, 2 (1993), 1847-1851. Conjugation of HBsAg DNA to the HBsAg peptide mixture was performed using benzoquinone. To 2mg/ml peptide in PBS
5 was added 1/10th final volume of *p*-benzoquinone (Sigma, B-1266) at 30mg/ml in 100% ethanol and 1/10th final volume of 1M potassium phosphate buffer, pH6. The mixture was incubated for 1 hour at room temperature in the dark and then passed through a Sephadex G50M column (Pharmacia, Milton Keynes) which had been presaturated in 1%
10 BSA in 0.15M NaCl. Elutions were monitored at 410nm and the initial peak containing the activated peptide was collected. To this was added equimolar HBsAg DNA and 1/10th final volume of 1M sodium carbonate/bicarbonate buffer pH 8.7 was added. The mixture was incubated with slow rotation at 4 °C in the dark for 48 hours. To remove
15 unconjugated peptides, the sample was then passed through a Bio-Gel HTP column (DNA grade, Biorad, Hemel Hempstead) and eluted with PBS according to the manufacturer's instructions. The resultant sample was then run on a 0.8% agarose gel and the peptide-DNA conjugate was purified using an Elutip cartridge (Schleicher and Schuell, Dassel,
20 Germany) according to the manufacturer's instructions, whereby molten gel slices containing DNA are applied to the Elutip column such that the matrix binds the DNA which is subsequently washed off in high salt buffer. The purified conjugate was then passed through a Sepharose 6HR FPLC column run in PBS and the leading fraction containing the
25 conjugate was collected and stored at -20 °C prior to experimentation. Control conjugates included either irrelevant DNA (pRc/CMV plasmid DNA, Invitrogen) or irrelevant peptide (YMNGTMSQV, from human tyrosinase).

For analysis of immune responses, 50 μ l samples of hypertonic sucrose (25%w/v in PBS) were injected bilaterally into the tibialis anterior muscles of 6 week-old anaesthetised BALB/c mice. 30 minutes later, 100 μ g samples of HBsAg DNA/peptide conjugate or 100 μ g HBsAg DNA or 100 μ g peptide, each in 50 μ l PBS, were injected into the same sites.

For analysis of MHC class I-mediated responses, mice were sacrificed 7 days after injection and 3×10^7 spleen cells from the mice were mixed with 10^8 peptide-pulsed and mitomycin C (Sigma)-treated spleen cells (10 μ g/ml synthetic HBsAg peptide added to 10^7 /ml syngeneic spleen cells and incubated for 3 hours at 37 °C) in 20ml Iscove's modified Dulbecco's medium (Gibco) with 10% foetal calf serum (Gibco), 50 μ M β -mercaptoethanol and 20units/ml recombinant IL-2 (R and D Systems, Oxford). Cells were incubated for 7 days at 37 °C and then used to challenge ^{51}Cr -labeled mouse A20 (TIB208) target cells (obtained from ATCC) pulsed with the MHC class I peptide at 10 μ g/ml for 1 hour at 37 °C. Serial dilutions of effector cells were incubated with 2×10^3 target cells in 200 μ l medium for 4 hours at 37 °C. 100 μ l of culture supernatants and the resuspended cell pellet were then counted and the data is shown in figure 1. This shows that the peptide-DNA conjugates gave a much higher CTL response to the MHC class I peptide than DNA or peptide alone or than an irrelevant peptide-HBsAg DNA conjugate indicating that the combination of synthetic peptide and peptide encoded by DNA produces an enhanced response.

For analysis of MHC class II-mediated responses, blood was collected from anaesthetised mice various times after injections by retrobulbar

puncture into heparinised glass pipettes, the blood was centrifuged and the serum stored at -20 °C until analysis. Anti-HBsAg antibodies were measured using the AUSAB EIA test (Abbott) according to the manufacturer's instructions and the results are shown in figure 2. This shows that the peptide-DNA conjugate ("HBPep:HBDNA") gave a much higher anti-HBsAg response to the MHC class I peptide than DNA alone ("HBDNA") or peptide alone ("HBPep") or than an irrelevant peptide-HBsAg DNA conjugate ("TyrPep:HBDNA") indicating that the combination of synthetic peptide and peptide encoded by DNA produces an enhanced response.

Example 2:

The study of example 1 was repeated except that individual conjugates with either the MHC class II-restricted HBsAg peptide or the MHC class I-restricted HBsAg peptide were made. CTL and antibody responses were measured as above and the results are shown in table 1. This shows that the peptide-DNA conjugates ("HBsAg class II-HBsAgDNA" and "HBsAg class I-HBsAgDNA") gave higher CTL and antibody responses than DNA or peptide alone or than an irrelevant peptide-HBsAg DNA conjugate.

Table 1

	Antibody -week 8 (mIU/ml)	CTL (% ⁵¹ Cr release)	
		E:T 10:1	E:T 5:1
HBSAg class II-HBSAg DNA	744	26	21
HBSAg class I-HBSAg DNA	260	42	44
Tyr Pep-HBSAg DNA	197	37	34
HBSAg DNA only	245	27	22
HBSAg class II peptide only	18	1	1
HBSAg class I peptide only	3	13	8

Example 3:

A monoclonal antibody directed to mouse MHC class II molecules was used to target an HBsAg DNA/MHC class I-restricted HBsAg peptide conjugate as described in example 2. The method was as for example 1 except that the rat monoclonal antibody B21-2 (ATCC TIB-229) was mixed with HBsAg or control peptide at a 1:1 molar ratio and co-conjugated to HBsAg DNA. Alternatively, B21-2 antibody was conjugated directly to DNA using the same conjugation conditions as for peptides. The antibody conjugates were purified using a Protein G column (Hi-Trap, Pharmacia). The column was washed with 5 column volumes of "Start Buffer" (20mM sodium phosphate pH7), the conjugate was applied and the column was washed with 5 volumes of Start Buffer to remove unbound HBsAg DNA and peptides. The sample was eluted with 0.1M glycine.HCl pH2.7 into 100μl 1M Tris pH8 and fractions containing conjugate were identified by absorbance at 280nm. To remove

unconjugated antibody, the sample was then passed through a Bio-Gel HTP column as in example 1.

MHC class I-mediated responses were measured as in example 1. The results as shown in table 2 indicate that conjugates of the B21-2 antibody with HBsAg DNA ("AntiMHCII-HBsAg DNA") gave an enhanced CTL response compared to DNA alone and that the mixed conjugate of B21-2 antibody with HBsAg class I peptide and HBsAg DNA ("AntiMHCII-HBsAg class I-HBsAg DNA") gave an additional further enhancement of CTL activity (although this was significant only at the 5:1 E:T ratio). This example illustrates that an antibody which targets DNA and peptides to APCs can enhance the induction of CTLs.

Table 2

	CTL (% ⁵¹ Cr release)	
	E:T 10:1	E:T 5:1
AntiMHCII-HBSAg class I-HBSAg DNA	50	46
AntiMHCII-HBSAg DNA	45	36
AntiMHCII-Tyr Pep-HBSAg DNA	38	30
HBSAg DNA only	26	21
HBSAg class I peptide only	5	8
AntiMHCII only	1	0

Example 4:

Human dendritic (DC) cell APCs were used as recipients for the experimental protein-DNA conjugates. The source of DC cells was buffy coat fractions from blood of normal volunteers which were first

fractionated on Ficoll-Hypaque gradients to enrich for peripheral blood mononuclear cells (PBMLs). The PMBL fraction was then added to a discontinuous Percoll gradient using steps of 30%, 40%, 50% and 75% using Dulbecco's PBS with 5% human serum as diluent from a 1.129 g/ml stock. After centrifugation at 1100g for 20 minutes at 4 °C, the 75% fraction was collected and diluted to 10^7 cells/ml in RPMI with 10% pooled human serum. Cells were incubated overnight in Teflon vessels and then further enriched for DC cells using the method of Markowicz and Engleman, *J Clin Invest* **85** (1990), 955).

10

For the protein-DNA vaccine molecules, purified recombinant human tyrosinase protein was produced by PCR cloning the tyrosinase gene into a mammalian expression vector and transfecting COS-7 or CHO cells using methods described in Topalian *et al.*, *Proc Natl Acad Sci USA*, **91** (1994), 9461-9465. This was conjugated to DNA encoding the tyrosinase gene using the method of Poncet *et al* (*ibid*).

The tyrosinase-DNA conjugate and control of tyrosinase and DNA alone were first incubated with HLA-A2⁺ or HLA-DR4⁺ human dendritic cells prepared as above. Cell controls comprised either HLA-A2⁻ and HLA-DR4⁻ dendritic cells or total HLA-A2⁺ or HLA-DR4⁺ human peripheral blood lymphocyte preparations. Cells were then washed and then incubated with lymphocytes from HLA-A2⁺ or HLA-DR4⁺ naive donors. For cytotoxic T cell assays, ⁵¹chromium-labelled tyrosinase⁺ human melanoma cells were added and release of ⁵¹chromium was measured. For T helper activity assays, human IL-2 was added to the dendritic cell-lymphocyte mixture followed by ³H-thymidine and uptake of this radiolabel was measured. The results showed that the tyrosinase-DNA

conjugate incubated with HLA-A2⁺ or HLA-DR4⁺ dendritic cells gave increased CTL and T helper responses with HLA-A2⁺ and HLA-DR4⁺ lymphocytes respectively compared to tyrosinase alone or DNA alone which gave predominantly T helper and CTL responses respectively. Also,
5 minimal responses were obtained with HLA-A2⁻ or HLA-DR4⁻ dendritic cells or lymphocytes.

Example 5:

The study of example 1 was repeated except that recombinant tyrosinase
10 protein was substituted by the conjugation of peptides from the tyrosinase protein comprising the class I-restricted epitope MLLAVLYCL and the class II-restricted epitope QNILLSNAPLGPQFP. The mixed peptide-DNA molecule gave rise to CTL activity with HLA-A2⁺ dendritic cells, lymphocytes and target cells and to T helper activity with HLA-DRB1⁺
15 cells.

Example 6:

The molecules of example 1-5 are formulated as vaccines by preparation in saline solution using DMSO to solubilise when necessary. These
20 preparations are injected into humans directly or administered as an aerosol via the lungs. In order to enhance the presence of APCs in the vicinity of the site of injection, cytokines such as GM-CSF or other treatments are administered either with the injection, before the injection, or both before and after the injection. Typical injected doses are in the
25 range of 10 nmoles to 1 μ mole of the molecule, and the vaccine is administered on multiple occasions, for example weekly for a period of four weeks or longer if required to achieve the desired immunological or therapeutic effect.

Example 7:

For *ex vivo* administration of the molecules of examples 1-5, human dendritic cells are isolated using magnetic beads as described in the publication "MACS and more" (vol 1, no 1 (1997), Miltenyi Biotech, Bergisch Gladbach, Germany), and cultured in cytokines selected from GM-CSF, TNF α and IL-4, depending on the source of dendritic cells. The vaccine molecules are added to the cultures in a dose range typically between 1 μ M to 1 mM. The cultures are continued for a period of 1 day, in order for the dendritic cells to take up and process the vaccine and are then injected back into the patients. The cultures can be washed prior to injection in order to remove excess vaccine molecules.

Example 8:**15 Antigen-Encoding DNA Complexed with Anti-DNA Antibody**

DNA encoding Hepatitis B surface antigen (HBsAg) was reacted with a ten fold molar excess of murine anti-DNA antibody (MCA1153, Serotec, Oxford, UK). For DNA encoding HBsAg, the vector pCMV-HBs was recreated as described by Davis et al., Human Molecular Genetics, 2 (1993), 1847. Reaction of the HBsAg with the antibody was performed in PBS at 37°C for 1 hour. Unreacted antibody was removed by gel filtration on a Superose 6HR FPLC column (Pharmacia, Milton Keynes, UK)) using the manufacturer's instructions. The first eluted fractions contained the Ab-DNA and the free Ab was eluted in the last fractions.

For analysis of immune responses, 50 μ l samples of hypertonic sucrose (25% w/v in PBS) was injected intra-dermally into triplicate 6 week old

BALB/c mice. After 30 minutes, 100µg samples of HBsAg DNA/Ab or 100µg HBsAg DNA or 100µg Ab, each in 50µl PBS, were injected into the same sites.

- 5 For analysis of MHC class I mediated immune responses, mice were sacrificed 7 days after injection and 3×10^7 spleen cells from the mice were mixed with 10^8 peptide-pulsed and mitomycin C (Sigma)-treated spleen cells (10µg/ml synthetic HBsAg peptide added to 10^7 /ml syngeneic spleen cells and incubated for 3 hours at 37°C) in 20ml Iscoves modified
- 10 Dulbeccos medium (Gibco) with 10% foetal calf serum (Gibco), 50µM mercaptoethanol and 20 units/ml recombinant IL-2 (R and D Systems, Oxford). Cells were incubated for 7 days at 37°C and then used to challenge ^{51}Cr -labeled mouse A20 (TIB208) target cells (obtained from ATCC) pulsed with the MHC class I peptide at 10 µg/ml for 1 hour at 37°C.
- 15 Serial dilutions of effector cells were incubated with 2×10^3 target cells in 200 µl medium for 4 hours at 37°C. 100µl of culture supernatants and the resuspended cell pellet were then counted. The results were analysed by ^{51}Cr release from target cells and the results are expressed as the percentage of total ^{51}Cr released by the target cells. A detectable response
- 20 to the molecule of the invention is held to exist if the percentage of ^{51}Cr released is significantly increased where the molecule of the present invention is tested with target cells pulsed with a peptide related to a component of the molecule when compared to the percentage released where the molecule of the present invention is not added or where the
- 25 target cells are pulsed with an unrelated peptide. As shown in figure 3, a detectable MHC class I-mediated response was observed for a range of ratios of effector spleen cells : target A20 cells (effector:target) in mice injected with HbSAg DNA (HBDNA) complexed with anti-DNA antibody

(Mab) when compared with DNA alone (HBDNA only), anti-DNA antibody only (Mab only) or a negative control anti-DNA antibody complexed with an irrelevant pSVgpt plasmid DNA (Mab-SVDNA).

- 5 For analysis of MHC class II-mediated immune responses, blood was collected from anaesthetised mice various times after injection by retrobulbar puncture into heparinised glass pipettes, the blood centrifuged and the serum stored at -20°C until analysis. Anti-HBsAg antibodies were measured using AUSAB EIA test (Abbott) according to the manufacturer's
- 10 instructions. A biologically significant difference in response is held to exist if the difference in response measured in the above assays is equal to or greater than that known from the art to equate to a biologically significant difference in response to the intended antigen in that mammal. As shown in figure 4, a biologically significant MHC class II-mediated
- 15 response was observed in mice injected with HbSAg DNA/Ab compared to various controls with antibody levels measured 4, 6 and 8 weeks after injection.

Example 9:

20

- The DNA/antibody molecule of example 8 is formulated as a vaccine by preparation in saline solution. The preparation is injected into humans directly or administered as an aerosol via the lungs. In order to enhance the presentation of APCs in the vicinity of the site of injection, cytokines
- 25 such as GM-CSF or other treatments are administered either with the injection, before the injection or both before and after the injection. Typical injected doses are in the range of 10 nmoles to 1 mole of the molecule, and the vaccine is administered on multiple occasions, for

example weekly for a period of four weeks or longer if required to achieve the desired immunological or therapeutic effect.

Example 10:

**5 Antigen-Encoding DNA molecules chemically attached to Antibody
Production of antibody-DNA molecules based on anti-idiotypic
Mab708**

**10 *Construction of variable heavy and variable light chain molecules of
antibody 708.***

All variable heavy chain (V_h) and variable light chain (V_k) molecules were assembled by overlapping PCR recombination. V_h molecules were assembled from three separate primary PCR products and joined into a single contig in a secondary PCR using flanking primers. V_k molecules
15 were assembled from two separate primary PCR products, and also joined into a single contig in a secondary PCR using flanking primers.

Method for assembly of humanised variable heavy chain:

Three separate primary PCR reactions were carried out, 1.1, 1.2 and 1.3.
20 Primary reaction 1.1 was performed using 10pmol each primers vvhk1 [5'-
gcatgttgaccctgacgcaagcttatgaatatgcaaa] and vh276r [5'-
gttgacgttgacacctcgagtgacacacctgtg] and 100ng rfDNA from M13 vector
VHPCR1 (Orlandi *et al* (1989), *Proc. Natl. Acad. Sci. USA*, 86: 3833-
3837) as template. The product of this reaction encompasses the first
25 276bp for the 5' end of the final contig.

Primary reaction 1.2 was carried out using a set of overlapping oligonucleotides; vhd1322f, vhd1446f, vhd1570f, vhd1340r, vhd1463r,

vhdt587r, vkdt570f (oligonucleotide sequences given below) each included in the reaction mix at a concentration of 1pmol. The reaction was driven by primers vh261f [5'-gaggtccagctgcaacagtc] and vh611r [5'-tgcagagacagtgaccaga] each present in the reaction mix at a concentration of 10pmol, to yield a 350bp product encompassing nucleotides 261-611 of the final contig.

Oligonucleotides for 708 vh primary reaction 1.2 have the following sequences:

10

vhdt340r:

5'tatccagaagtcttacaggatatcttcactgaagccccaggcttcaccagctcaggtccagactgttgca
gctggacctc

15

vhdt322f:

5'cctgtaagacttctggatacacattcactgaataacaacatgaactgggtgaggcagagccccggacag
agccttgagtgg

vhdt446f:

20

5'gaagttcaggggcaaggccacattgactgtagacaagtcctccagcacagcctacatggagctccgc
agcctgacatctg

vhdt463r:

25

5'gccttgcccctgaacttctggtgtagatagaaccaacattgttaggattaatacctccaatccactcaag
gctctgtcc

vhdt570f:

5'gtggcttactggggccaagggactctggtcactgtctctgca

vhdt587r:

5'ttgccccagtaagccacgtagttaccatagcctcttgacagtaatagactgcagaatcctcagatgtc
aggctgcgga

5

Primary reaction 1.3 was carried out using 10pmol each of primers vh597f [5'-gtcactgtctctgcaggtgagtccttacaac] and vh12 [5'-gcgatagctggactgaatggatcctataaatctctg] and 100ng VHPCR1 rDNA as previously. The product of this reaction encompasses the final 234bp at the 3' end of the contig.

10

The complete contig was assembled by mixing 1µl of the products of reactions 1.1, 1.2 and 1.3, and performing PCR in the presence of 10pmol each of primers vvhk1 and vh12. The product of this secondary or joining reaction was 839bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end. In all cases PCR was carried out in a final volume of 50µl using 0.75µl Expand High Fidelity DNA polymerase (Boehringer, Lewes, UK) and a reaction buffer containing 1.5mM MgCl supplied by the manufacturer. Deoxynucleotide triphosphates (Life Technologies, Paisley, UK) were used at a final concentration of 30µM.

15

Reactions were each pre-incubated at 96°C for 5 minutes, then cycled for a total of 20 cycles with the following parameters; 94°C for 30 seconds; 50°C for 30 seconds; 72°C 90 seconds. Reactions were completed by a final incubation at 72°C for 5 minutes. All oligonucleotides were purchased from GenoSys Biotechnologies (Cambridge UK).

20

Oligonucleotides greater than 35 nucleotides in length were supplied PAGE purified, others de-salted.

25

Method for assembly of humanised variable light chain:

Two separate primary PCR reactions were carried out, 1.1 and 1.2. Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and vk275r [5'ggtcacacaaatgtcggagtggacacctgt], and 100ng rfDNA from M13 vector VKPCR1 (Orlandi et al 1989, Proc. Natl. Acad. Sci.USA, 86: 3833-3837) as template. The product of this reaction encompasses the first 293bp for the 5' end of the final contig. Other components and cycle parameters were as described above.

Primary reaction 1.2 was carried out using a set of overlapping oligonucleotides; vkdt340r, vkdt322f, vkdt463r, vkdt446f, vkdt587r, vkdt570f (oligonucleotide sequences given below), each included in the reaction mix at a concentration of 1pmol. The reaction was driven by primers vk261f [5'gacattgtgatgacctagt] and vk12 [5'gcgatagctggactgaatggatccaactgaggaagca] each included in the reaction mix at a concentration of 10pmol, to yield a 396bp product encompassing nucleotides 261-447 of the final contig. Other components and cycle parameters were as described above.

Oligonucleotides for 708 vk primary reaction 1.2 have the following sequences:

vkdt322f:

5'taacctgcaaggccagtcagaatgtgaataactaatgtacctgggtatcaacagaaaccagggcaatctc
ctcaatcactg

vkdt340r:

5'tgactggccttgacaggttatgctgacctgtctcctactgatgtggacacgaatgttgagactgggtca
tcacaatgtc

vkdt446f:

5'ctcaggcagtg gatctgggacagattcactctcaccatcagcaatgtgcagtctgaagactttgcagag
tattactgtc

5

vkdt463r:

5'ccagatccactgcctgagaagcgatcagggactccactgaatcggtaggatgccgagtaaatacagtga
ttgaggagattg

10 vkdt570f:

5'ctggagctgaaacgtgagtagaatttaaactttgcttcctcagttggatccgc

vkdt587r:

5'ctcacgtttcagctccagcttgggtcccaccaccgaacgtgcgcggatagctgttatattgctgacagtaa

15 tactctgcaa

The complete contig was assembled by mixing 1µl of the products of reactions 1.1 and 1.2, together with 10pmol each of primers vlvk1 and vk12. PCR was performed in a final volume of 50µl using other components and cycle conditions as previously. The product of this secondary or joining reaction was 689bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end.

25 *Method for assembly of humanised variable heavy chain containing CEA epitopes:*

Three separate primary PCR reactions were carried out, 1.1, 1.2 and 1.3. Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and

vh276r, and 100ng VHPCR1 rDNA as previously. The product of this reaction encompasses the first 276bp for the 5' end of the final construct.

Primary reaction 1.2 was carried out using a set of overlapping
5 oligonucleotides; vhdt340r, vhdt322f, vhcea463r, vhcea447f, vhcea586r,
vhcea570f (sequences listed below) each included in the reaction mix at a
concentration of 1pmol. The reaction was driven by primers vh261f and
vh611r2 [5'-tgcagagacagtgccag] at 10pmol each, to yield a 350bp product
encompassing nucleotides 261-611 of the final contig.

10

Oligonucleotides for 708 vhcea primary reaction 1.2 not listed previously
have the following sequences:

vhcea447f:

15 5'gcctccggcaccaaggccacattgactgtagacaagtcctccagcacagcctacatggagctccgca
gcgccacatctga

vhcea463r:

5'gccttggtgccggaggcggacacggtgatagacttaacgatggagtattgcgacctccgttcactca
20 aggctctgtcc

vhcea570f:

5'cgccccggcggtgggccaagggaactctgggcactgtctctgca

25 vhcea586r:

5'tggcccacgccggggcggtagtaggtataggagggggagatgtagatgcctgcagaatcctcagatg
tggcgctgcg

Primary reaction 1.3 was carried out using 10pmol each of primers vhcea597f [5'-ggcactgtcctctgcaggtgagtccttaca] and vh12 and 100ng VHPCR1 rfDNA as previously. The product of this reaction encompasses the final 234bp at the 3' end of the construct.

5

The complete contig was assembled by mixing 1µl of the products of reactions 1.1, 1.2 and 1.3, and performing PCR in the presence of 10pmol each of primers vlvk1 and vh12. The product of this secondary or joining reaction was 839bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end. PCR was carried out in a final volume of 50µl using reagents and conditions as described above.

10

Method for assembly of humanised variable light chain containing CEA epitopes:

Two separate primary PCR reactions were carried out, 1.1 and 1.2. Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and vk275r and 100ng VKPCR1 rfDNA template as previously. The product of this reaction encompasses the first 293bp at the 5' end of the final contig.

20

Primary reaction 1.2 was carried out using a set of overlapping oligonucleotides; vkcea324f, vkcea340r, vkcea450f, vkcea486r, vkcea576f, vkcea592r (oligonucleotide sequences given below) each included in the reaction mix at a concentration of 1pmol. The reaction was driven by primers vk261f and vk12 at 10pmol each, to yield a 396bp product encompassing nucleotides 261-447 of the final contig.

25

Oligonucleotides for 708 vkcea primary reaction 1.2 have the following sequences:

vkcea324f:

5 5'acctgcaccctgctgtccgtgacccgcaacgacgtatcccgctatcaacagtccccagggcaatggcc
tcaatcactgat

vkcea340r:

10 5'gacagcaggggtgcaggtcacgctggcggagtctcctactgatgtggacacgaattttgagactgggt
catcacaatgtc

vkcea450f:

15 5'ggcagtggatctgggacagatttcactctcaccatcagcaatgtgcagtctgaagacctggcagagttc
atgtgttacct

vkcea486r:

5'gtcccagatccactgcctgagaagcgatcagggactccactgaatcggtaggatgccgagtaaatacag
tgattgaggcca

20 vkcea576f:

5'gtgcgacgtgagtagaatttaaactttgcttcctcagttggatccgc

vkcea592r:

25 5'ttctactcacgtcgacctccagcttgggtcccaccaccggtcaggttcaggttggcgccggacaggtaa
cacatgaactc

The complete contig was assembled by mixing 1µl of the products of reactions 1.1 and 1.2, and performing PCR in the presence of 10pmol each

of primers vlvk1 and vk12. The product of this secondary or joining reaction was 689bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end. PCR was carried using reagents and conditions described above.

5

Method for assembly of humanised variable heavy chain containing CEA and CD55 epitopes:

Three separate primary PCR reactions were carried out, 1.1, 1.2 and 1.3. Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and
10 vlv276r, and 100ng VHPCR1 rfDNA as previously. The product of this reaction encompasses the first 276bp at the 5' end of the final contig.

Primary reaction 1.2 was carried out using a set of overlapping oligonucleotides vlvcd322f, vlvcd340r, vlvcd463r, vlvea447f, vlvea570f,
15 vlvea586r, each included in the reaction mix at a concentration of 1pmol. The reaction was driven by primers vlv261f and vlv611r2 at 10pmol each, to yield a 350bp product encompassing nucleotides 261-611 of the final contig.

20 Sequences of oligonucleotides vlvcd340r and vlvcd322f are as below. Other oligonucleotides required for primary reaction 1.2 are as given previously:

vlvcd340r:

25 5'tttagccggtgttcaggagaaggagatgggtggcgccgaacttcgccagctcggggccggactgct
gcagctgcacctc

vlvcd322f:

5'ctgcaacaccggctacaagctgttcggctccacctccatgaaccgacttcgccagtcccccgccagt
ccctggagtggga

vhcd463r:

5 5'gccttggtgccggaggcggacacgggtgatagacttaacgatggagtattgacacctccgtccactcc
agggactggcc

Primary reaction 1.3 was carried out using 10pmol each of primers
vhcea597f and vh12, and 100ng VHPCR1 rfDNA as previously. The
10 product of this reaction encompasses the final 234bp at the 3' end of the
contig.

The complete contig was assembled by mixing 1µl of the products of
reactions 1.1, 1.2 and 1.3, and performing PCR in the presence of 10pmol
15 each of primers vlvk1 and vh12. The product of this secondary reaction
was 839bp and contained single restriction sites for HindIII at the 5' end
and BamHI at the 3' end. In all cases PCR was carried out in a final
volume of 50µl using reagents and conditions as described above.

20 ***Method for assembly of chimaeric (mouse/human) variable heavy chain:***

Three separate primary PCR reactions were carried out, 1.1, 1.2 and 1.3.
Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and
vh276r, and 100ng VHPCR1 rfDNA as previously. The product of this
reaction encompasses the first 276bp for the 5' end of the final contig.

25

Primary reaction 1.2 was carried out using a set of overlapping
oligonucleotides; vhch355r, vhch337f, vhch525r, vhch507f
(oligonucleotide sequences are as given below), each included in the

reaction mix at a concentration of 1pmol. The reaction was driven by primers vh261f and vh611r at 10pmol each, to yield a 350bp product encompassing nucleotides 261-611 of the final contig.

- 5 Oligonucleotides for 708 chimaeric vh primary reaction 1.2 have the following sequences:

vhch355r:

5'tattcagtgaatgtgtgtccagaagtcttacaggatatcttcactgaagccccaggcttcaccagctcag
10 gtccagactgttgacgtggacctc

vhch337f:

5'gacacacattcactgaatacaacatgcagtgggtgaagcagagccttgacagagccttgagtggatt
ggaggtattaatcctaacaatgttggttctatctac
15

vhch525r:

5'cagatgtcaggctgcggagctccatgtaggctgtgctggaggactgtctacagtcaatgtggccttgc
ccctgaacttctggtttagatagaaccaacatt

20 vhch507f:

5'ctccgcagcctgacatctgaggattctgcagtctattactgtgcaagaggctatggtaactacgtggctt
actggggccaagggactctggtcactgtctctgca

- Primary reaction 1.3 was carried out using 10pmol each of primers vh597f
25 and vh12, and 100ng VHPER1 rDNA as previously. The product of this reaction encompasses the final 234bp at the 3' end of the contig.

The complete contig was assembled by mixing 1µl of the products of reactions 1.1, 1.2 and 1.3, and performing PCR in the presence of 10pmol each of primers vlvk1 and vl12. The product of this secondary or joining reaction was 839bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end. PCR was carried out in a final volume of 50µl using reagents and conditions as described above.

Method for assembly of chimaeric (mouse/human) variable light chain:

Two separate primary PCR reactions were carried out, 1.1 and 1.2. Primary reaction 1.1 was performed using 10pmol each primers vlvk1 and vk275r and 100ng VKPCR1 rDNA as previously. The product of this reaction encompasses the first 293bp at the 5' end of the final contig.

Primary reaction 1.2 was carried out using a set of overlapping oligonucleotides; vkch364r, vkch345f, vkch533r, vkch518f (oligonucleotide sequences are as given below), each included in the reaction mix at a concentration of 1pmol. The reaction was driven by primers vk261f [5'gacattgtgatgaccagt] and vk12 at 10pmol each, to yield a 396bp product encompassing nucleotides 261-447 of the final contig.

Oligonucleotides for 708 chimaeric vk primary reaction 1.2 have the following sequences:

vkch345f:

5'gtgaataactaatgtagcctggatcaacagaaaccagggaatctcctaaatcactgattactcgcat
cctaccgatacagtgaggatccctgatcgcttcac

vkch364r:

5'caggctacattagtagttcacattctgactggccttgcaggtgacgctgaccctgtctcctactgatgtgg
acatgaattttgagactgggtcatcacaatgtc

vkch518f:

5 5'tttctgtcagcaatataacaggtatccgttcacgttcggtgggtgggaccaagctggagctgaaacgtgag
tagaatttaaactttgcttctcagtggatccgc

vkch533r:

5'atattgctgacagaaaaactctgccaaagtcttcagactgcacattgctgatggtagagtgaaatctgtc
10 ccagatccactgcctgtgaagcgatcagggactc

The complete contig was assembled by mixing 1µl of the products of reactions 1.1 and 1.2, and performing PCR in the presence of 10pmol each of primers vvhk1 and vk12. PCR was performed in a final volume of 50µl
15 using other components and cycle conditions as previously. The product of this secondary or joining reaction was 689bp and contained single restriction sites for HindIII at the 5' end and BamHI at the 3' end.

Method for Cloning of PCR Assembled vh & vk constructs for sequence analysis:
20

Secondary PCR products were digested with restriction enzymes HindIII and BamHI (Boehringer, Lewes UK) and cloned into pUC19 vector using standard techniques (Molecular cloning: a laboratory manual, eds. Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory Press,
25 1989 New York, USA). Recombinant clones in *E.coli* strain TG1 were selected for full sequence analysis. Plasmid DNA was prepared using the Wizard SV system and instructions provided by the manufacturer (Promega, Southampton UK). DNA was sequenced from both directions

using primers - 40FOR [5'-gttttccagtcagtcacgacgttgta] and -50REV [5'-
ttgtgagcggataacaatttc]. Some reactions were primed using internal primers
vh261f and vk261f. In all cases sequencing reactions were performed
using 33P-labelled dideoxynucleotide terminators and thermosequase
5 using conditions recommended by the supplier (Amersham, UK).

Method for expression and Purification of Variant 708 molecules:

Satisfactory Vh clones were sub-cloned using HindIII and BamHI
(Boehringer, Lewes, UK) as previously, into mammalian expression
10 vector pSVgptVHHuIgG1 containing a human immunoglobulin IgG1
constant region gene (Orlandi *et al* (1989), *Proc. Natl. Acad. Sci.USA*, 86:
3833-3837). Satisfactory Vk constructs were sub-cloned using HindIII
and BamHI sites into mammalian expression vector pSVhygVKHuCK
containing a human immunoglobulin kappa constant region gene (Orlandi
15 *et al* (1989), *Proc. Natl. Acad. Sci.USA*, 86: 3833-3837). Selected clones
were grown and plasmid DNA prepared as previously. Combinations of
heavy and light chain construct plasmid DNA was mixed in a mass ratio
of 1:2 and a total of 10µg total plasmid DNA co-transfected into NSO
mouse myeloma cells (ECCACC # 85110503) using electroporation (Gene
20 Pulser, BioRad, UK). Mycophenolic acid resistant clones were selected
and clones screened for antibody production as described previously
(Tempest *et al*, (1991), *Bio/Technology* 9: 266-271). Antibody was
purified by protein A affinity chromatography, and analysed by SDS-
PAGE under reducing conditions using standard techniques (Antibodies: a
25 laboratory manual. eds, Harlow & Lane, Cold Spring Harbor Laboratory
Press, 1988 New York, USA).

Method for producing a "CEA-mini-gene" construct for 708 antibody

coupling:

A mini-gene construct capable of directing the expression of CEA peptides was assembled. An overlapping PCR recombination technique was used to produce a CEA mini-gene, which was then inserted into an
5 expression vector capable of directing expression in mammalian cells.

Oligonucleotides CMG80R, CMG63F, CMG198R, CMG181F, (oligonucleotide sequences given below) at a concentration of 1pmol were mixed with 18-mer oligonucleotide primers CMG1FS [5'-
10 gcatgttgaccctgacgc] and CMG237RS [5'- agtcagtcggaacattct] each at a concentration of 10pmol. Deoxynucleotide triphosphates (Life Technologies, Paisley, UK) at 30µM, PCR reaction buffer containing 1.5mM MgCl (Boehringer, Lewes, UK) were added and the reaction mixture brought to a total volume of 50µl. 0.75µl Expand High fidelity
15 DNA polymerase (Boehringer, Lewes, UK) was added, and the reaction pre-incubated at 96°C for 5 minutes, then cycled for a total of 20 cycles with the following parameters; 94°C for 30 seconds; 50°C for 30 seconds; 72°C 90 seconds. The reaction was completed by a final incubation at 72°C for 5 minutes. All oligonucleotides were purchased from GenoSys
20 Biotechnologies (Cambridge UK), oligonucleotides greater than 35 nucleotides in length were supplied PAGE purified, others de-salted. The 237bp PCR product was verified by agarose gel electrophoresis and purified by phenol extraction before digestion with restriction endonucleases EcoRI and Xba I (Boehringer, Lewes, UK) and cloning into
25 EcoRI, XbaI prepared pUC19 vector according to standard techniques (Molecular cloning: a laboratory manual, eds. Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory Press, 1989 New York, USA).

Full sequence analysis of the CEA-mini-gene was carried-out on cloned DNA in vector pUC19 using primers and methods as described above.

Oligonucleotides for CEA-mini-gene assembly have the following sequences:

CMG80R:

5'gaggcggacacggtgatggacttcacgatggagttgttcggccggcgcccatgaattcgcgtc
agggtcaacatgc

CMG63F:

5'catcacgtgtccgcctccggcaccgccgcccatctccccctctacacctactaccgccccggcg
ccgccgccaccc

CMG198R:

5'caggttggcgccggacaggtaggcggcgccacgtcgttcgggtcacggacagcaggggtggcgg
cggcgccggggcggt

CMG181F:

5'ctgtccggcgccaacctgaacctggccgccgctaataatggttcggactgact

The completed CEA-mini-gene was sub-cloned into mammalian expression vector pCI (Promega, Southampton, UK), using restriction sites EcoRI and XbaI as previously. Clones were analysed for correct restriction enzyme pattern using standard techniques (Molecular cloning: a laboratory manual, eds. Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory Press, 1989 New York, USA), and a satisfactory clone selected for growth. Plasmid DNA preparation was carried out on a

500ml L-broth culture using a Qiagen-tip 500 system and protocols supplied by the manufacturer (Qiagen, Crawley, UK).

5 ***Method for coupling DNA "CEA mini-gene" construct to purified 708 antibody molecules:***

The coupling of the CEA mini-gene expression plasmid to a purified recombinant 708 antibody molecule (vhCEA/vkCEA) was achieved using p-benzoquinone essentially as described by Ternynck et al (Ternynck *et al*
10 (1976), *Ann. Immunol. (Inst Pasteur)*, 127: 197-208).

Briefly, purified antibody at a concentration of 2 mg/ml in PBS, was mixed with 10 volumes of p-benzoquinone (Sigma, Poole, UK, #B-1266) at 30 mg/ml in 100% ethanol and 1:10 final volume of 1M potassium-phosphate buffer pH 6.0. The mixture was incubated at room temperature
15 in darkness for 1 hour. The activated antibody was purified from free (un-reacted) p-benzoquinone using a Sephadex G25M column (Pharmacia, St Albans, UK) pre-saturated with 1% BSA in 150mM NaCl. The activated antibody fraction was detected by analysis of optical density at 410nm,
20 and eluted as a visibly pink eluate.

Plasmid DNA was coupled to the activated antibody by mixing activated antibody with DNA at a ratio of 10:1 (by weight) in a carbonate/bicarbonate pH 8.7 buffer solution of final concentration 0.1M.

25 The coupling reaction was allowed to proceed in the darkness by incubation at 4°C with slow rotation for 24 hours. Following incubation, un-coupled antibody was removed from the mixture by gel filtration using a Separose 6HR FPLC column (Pharmacia, St Albans, UK) and standard

conditions recommended by the supplier. The collected fraction was dialyzed against PBS and concentrated using Centricon 10 cartridges and protocols provided by the manufacturer (Amicon, MA, USA).

- 5 The combination of DNA and humanised 708 (vhCEA/vkCEA) antibody was shown to elicit CEA-specific MHC class I and class II-mediated responses. Class I assays were performed as described by Tsang (Tsang KY *et al* (1995) *J Natl Cancer Inst* 87, 982-990) and class II responses measured using methods according to Durrant (Durrant LG *et al* (1992) *Int J Cancer* 50, 811-816.

Method for assembly of humanised variable heavy chain containing alternative CEA epitopes:

- Three separate primary PCR reactions 1.1, 1.2 and 1.3 were carried out as previously. Primer combinations, oligonucleotides, DNA templates and reaction conditions were as given above for the assembly of the variable heavy chain containing CEA epitopes. However, an alternative CEA epitope (₂₈CEA₃₆) was introduced into CDRH3 by use of oligonucleotides vhcea586-2 and vhcea570-2 in place of oligonucleotides vhcea586r and
20 vhcea570f in primary reaction 1.2.

Oligonucleotides vhcea586-2 and vhcea570-2 have the following sequences:

- 25 Vhcea586-2:

5'- tggcctttgtaccatgagtatccgaataggtgccttgcacagtagatgcctgcagaatcctcagatgt
ggcgctgcg

vhcea570-2:

5'-tcatggtacaaaggccaagggactctgggcactgtctctgca

CLAIMS:

1. A molecule comprising 1) a nucleic acid portion from which at least one peptide for presentation on MHC class I or class II molecules, or both,
5 may be derived and 2) a polypeptide portion, from which at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived.
2. A molecule comprising 1) a nucleic acid portion from which at least
10 one peptide for presentation on MHC class I or class II molecules, or both, may be derived and 2) a polypeptide portion comprising a recognition domain capable of targeting the molecule to an antigen presenting cell (APC) wherein the polypeptide portion does not comprise a specific antigen binding site.
- 15 3. A molecule according to claim 2 wherein at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived from the polypeptide portion.
- 20 4. A molecule according to any one of claims 1 to 3 in which at least one of the peptides which may be derived from the nucleic acid portion, is associated with a disease or condition.
- 25 5. A molecule according to any one of claims 1, 3 or 4 in which at least one of the peptides which may be derived from the polypeptide portion is associated with a disease or condition.

6. A molecule according to any one of claims 1 to 5 in which the nucleic acid is DNA.

7. A molecule according to any one of claims 1 to 5 in which the nucleic acid is RNA.

8. A molecule according to any one of claims 1, 3 to 7 in which at least one of the peptides which may be derived from the nucleic acid portion has a region which is substantially identical to a region of at least one of the peptides which may be derived from the polypeptide portion.

9. A molecule according to claim 1 in which the molecule comprises a recognition domain.

10. A molecule according to any one of claims 2, 3 or 9 in which the polypeptide portion comprises an immunoglobulin molecule or a portion thereof.

11. A molecule according to claim 10 in which the immunoglobulin molecule or portion thereof comprises an Fc region and optionally does not comprise a V_H or V_L region or functional part thereof.

12. A molecule according to claim 10 or 11 wherein the said at least one peptide for presentation on MHC class I or class II molecules, or both, may be derived from the immunoglobulin molecule or portion thereof.

13. A molecule according to any one of claims 1 to 12 in which the nucleic acid portion also encodes a polypeptide for enhancing an immune response.

5 14. A molecule according to claim 4 or 5 in which the disease or condition is caused by an organism pathogenic to mammals.

15. A molecule according to claim 14 in which at least one of said peptides is immunologically cross-reactive with a peptide derivable from a
10 polypeptide found in the pathogenic organism.

16. A molecule according to claim 14 or 15 in which the disease or condition is caused by a virus.

15 17. A molecule according to claim 4 or 5 in which the disease or condition is cancer.

18. A molecule according to claim 17 in which the cancer is leukaemia, prostate cancer, breast cancer, lung cancer, liver cancer or bowel cancer.

20

19. A molecule according to claim 17 or 18 in which at least one of said peptides is immunologically cross-reactive with a peptide derivable from a polypeptide found in leukaemia, prostate cancer, breast cancer, lung cancer, liver cancer or bowel cancer cells.

25

20. A molecule according to claim 19 wherein the polypeptide is carcino-embryonic antigen (CEA) or CD55.

21. A molecule according to claim 4 or 5 in which the disease or condition is an autoimmune disease or condition.

22. A molecule according to claim 21 in which the disease or condition is
5 Grave's disease, multiple sclerosis, systemic lupus erythematosus, diabetes mellitus, Kawasaki's disease or rheumatoid arthritis.

23. A molecule according to claim 21 or 22 in which at least one of said peptides is immunologically cross-reactive with a peptide derivable from a
10 polypeptide found in the thyroid, myelin sheath cells, nucleoproteins, pancreatic islet cells, mucocutaneous lymph nodes, bones or synovial joints.

24. A molecule according to claim 4 or 5 in which the aetiology of the
15 disease or condition involves allergy.

25. A molecule according to claim 24 in which the disease or condition is atopic dermatitis, allergic rhinitis, allergic conjunctivitis, atopic asthma or eczema.

26. A molecule according to claim 24 or 25 in which at least one of said peptides is immunologically cross-reactive with a peptide derivable from a polypeptide found in pollen, animal dander, house dust mites or washing
20 detergent enzymes.

27. A molecule according to any of the preceding claims wherein the nucleic acid portion is bound to the polypeptide portion by means including a specific interaction between 1) a part of the polypeptide

portion that comprises an immunoglobulin domain that is capable of interacting specifically with the nucleic acid and 2) the nucleic acid.

28. A molecule according to any one of claims wherein the nucleic acid
5 portion is bound to the polypeptide portion by means including a specific interaction between 1) a part of the polypeptide portion that comprises an immunoglobulin domain that is capable of interacting specifically with a polypeptide that is bound to the nucleic acid 2) the polypeptide that is bound to the nucleic acid.

10

29. A molecule according to any one of claims 1-28 for use in medicine.

30. Use of a molecule according to any one of claims 1-28 for the manufacture of a medicament for treatment of a patient in need thereof.

15

31. Use of a molecule according to claim 17 for the manufacture of a medicament for treatment of a patient with cancer.

20

32. Use according to claim 31 in which the cancer is leukaemia, prostate cancer, breast cancer, lung cancer, liver cancer or bowel cancer.

33. Use of a molecule according to claim 21 for the manufacture of a medicament for treatment of a patient with an autoimmune disease or condition.

25

34. Use according to claim 33 in which the autoimmune disease or condition is Grave's disease, multiple sclerosis, Systemic Lupus

Erythrematosis(SLE), diabetes mellitus, Kawasaki's disease or rheumatoid arthritis.

35. Use of a molecule according to claim 16 for the manufacture of a
5 medicament for treatment of a patient with a viral disease or condition.

36. Use according to claim 35 in which the virus is HIV or Hepatitis B.

37. Use of a molecule according to claim 14 for the manufacture of a
10 medicament for treatment of a patient with a disease or condition caused
by a parasitic or symbiotic organism.

38. Use of a molecule according to claim 24 for the manufacture of a
15 medicament for treatment of a patient with a disease or condition in which
the aetiology of said disease or condition involves allergy.

39. Use according to claim 38 in which the disease or condition is
atopic dermatitis, allergic rhinitis, allergic conjunctivitis, atopic asthma or
eczema.

20 40. Use according to any one of claims 30 -39 in which the patient is also
treated with an immunological adjuvant.

41. A composition, characterised in that it comprises a molecule according
25 to any one of claims 1-28, together with a physiologically acceptable
carrier substance.

42. A method of vaccination comprising administering a composition according to claim 41 to a patient in need of such vaccination.

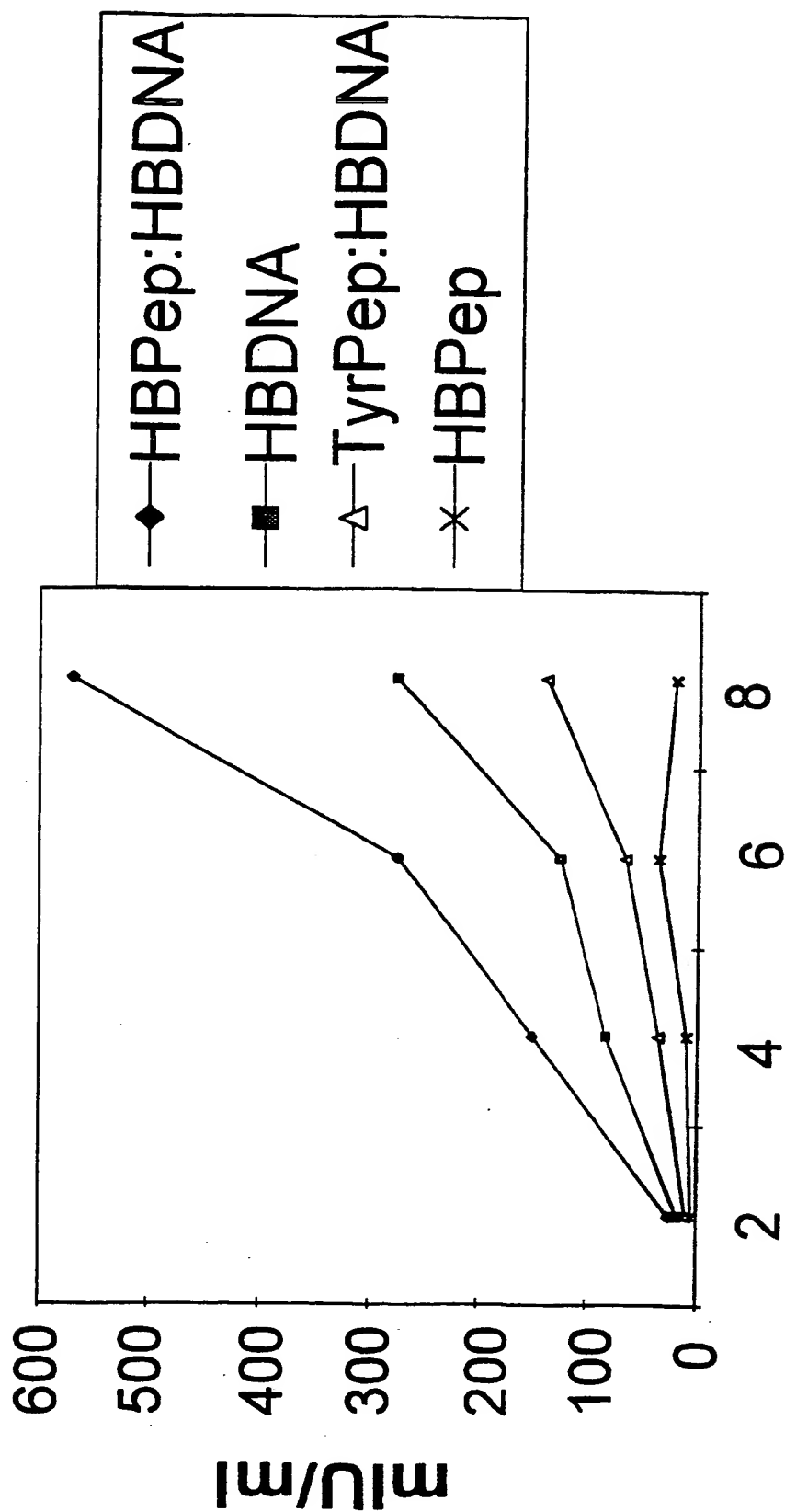
43. A method according to claim 42 in which the patient is also treated
5 with an adjuvant.

44. An *ex vivo* method of treatment of cells isolated from a patient consisting of administering a compound according to claims 1-28 to the cells.
10

45. A method according to claim 44 in which the cells are also activated *ex vivo* prior to reintroduction into the patient.

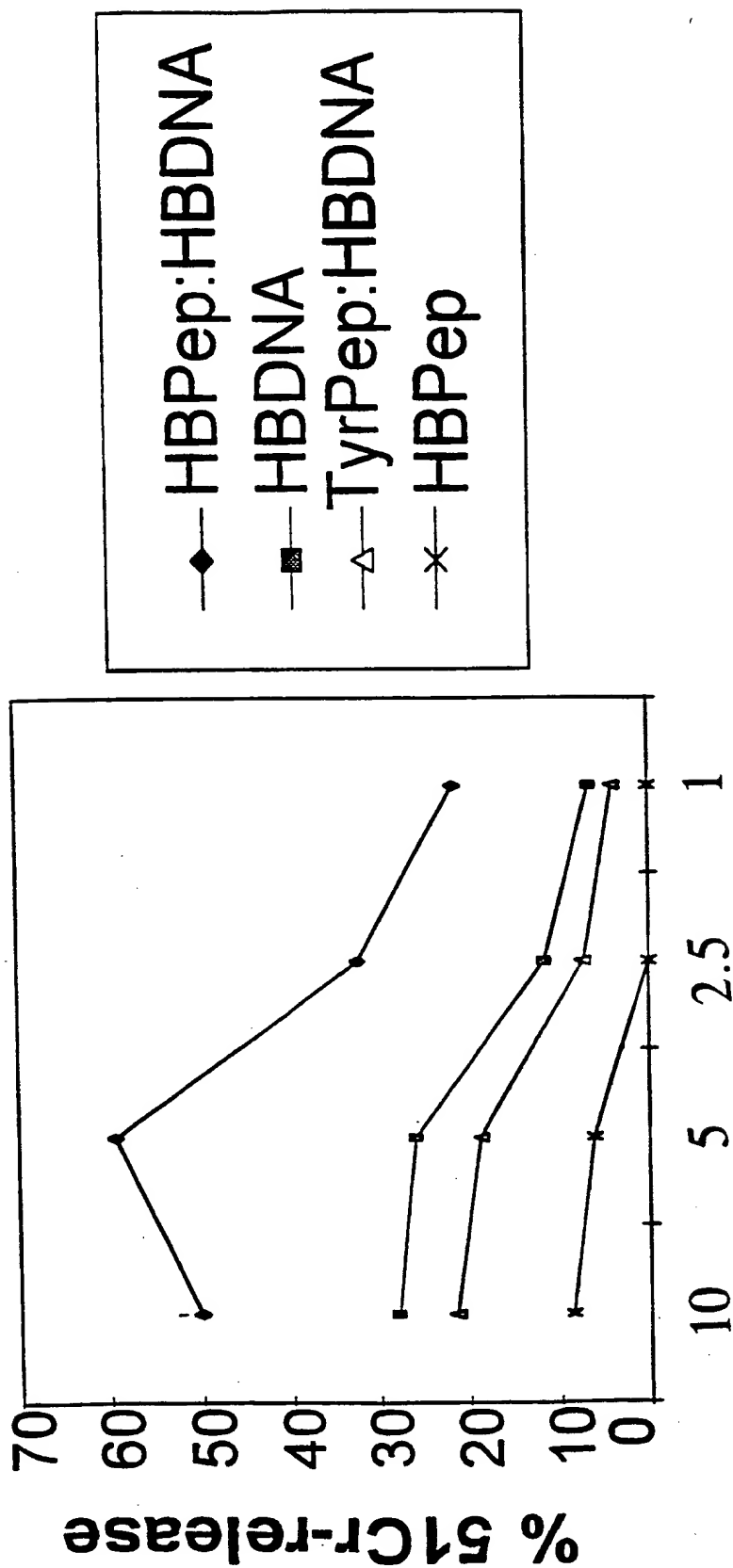
46. A method according to claim 44 or 45 in which the cells are antigen
15 presenting cells (APCs), including dendritic cells.

47. A method of making a molecule according to any one of claims 1-28 wherein the polypeptide portion is attached to the nucleic acid portion.



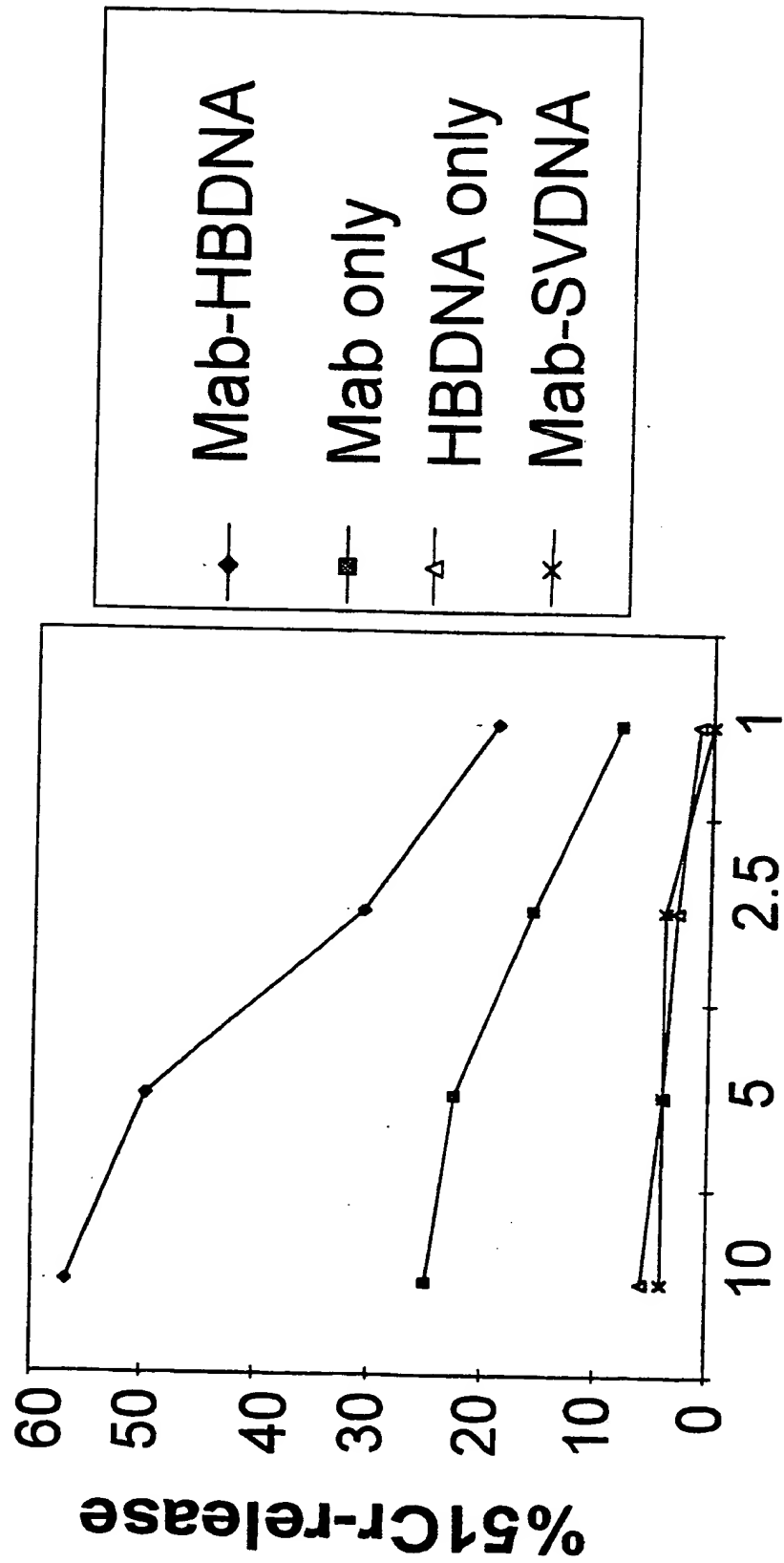
weeks after injection

FIGURE 1



Effector:target ratio

FIGURE 2



Effector:target ratio

FIGURE 3

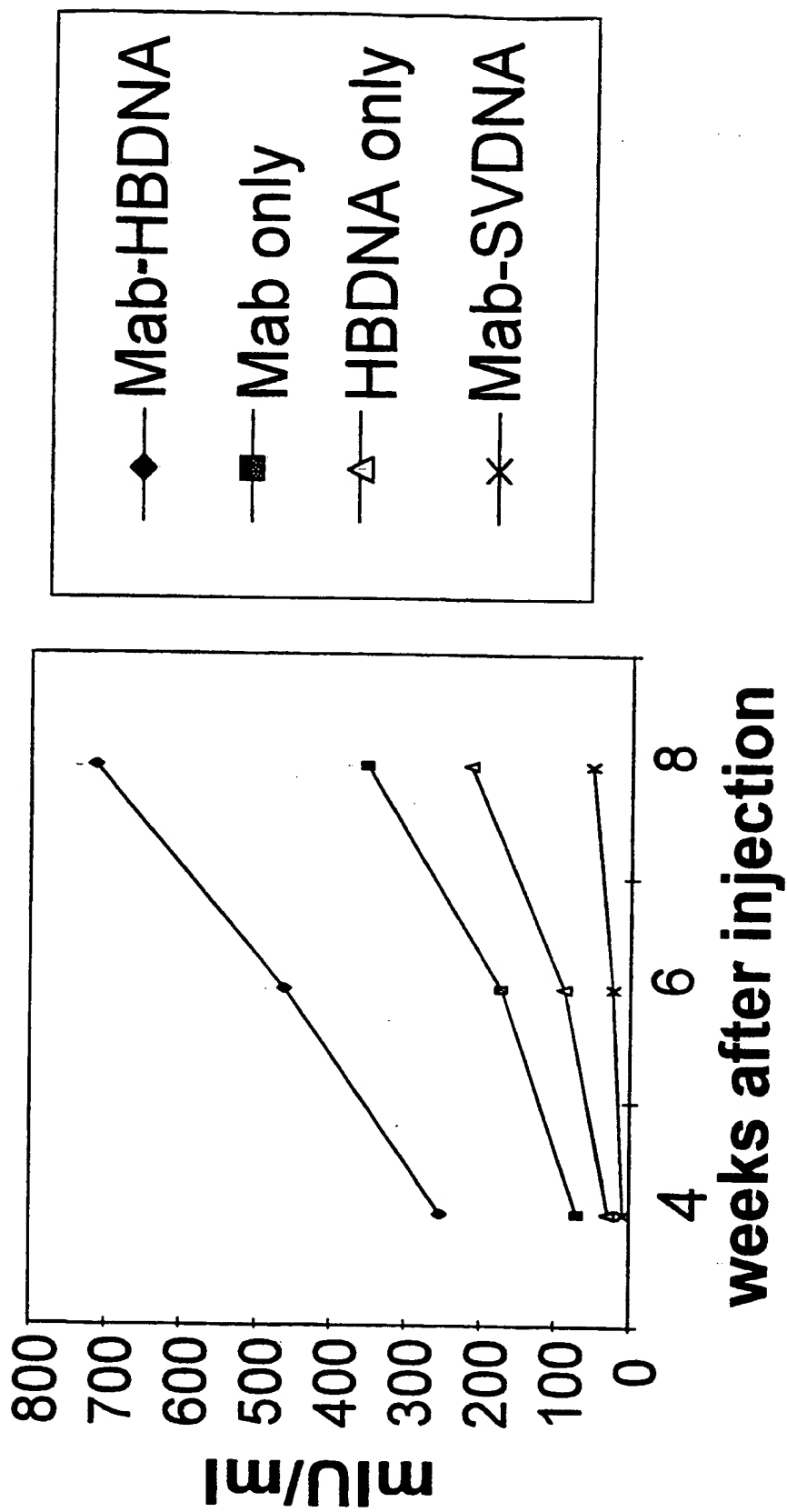


FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00325

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/385 C07K14/00 C12N15/11 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05399 A (UNIV CALIFORNIA) 23 February 1995 see abstract and claims	1
X	--- PONCET P. ET AL.,: "Antifection: an antibody-mediated method to introduce genes into lymphoid cells in vitro and in vivo" GENE THERAPY, vol. 3, - August 1996 pages 731-738, XP002066617 cited in the application see the whole document	1
X	--- US 4 587 044 A (MILLER PAUL S ET AL) 6 May 1986 see description column 1 and 2 --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

2 June 1998

Date of mailing of the international search report

08. 07. 1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Müller, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00325

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ULMER J B ET AL: "HETEROLOGOUS PROTECTION AGAINST INFLUENZA BY INJECTION OF DNA ENCODING A VIRAL PROTEIN" SCIENCE, vol. 259, 19 March 1993, pages 1745-1749, XP002009751 see the whole document</p> <p>---</p>	1-47
A	<p>FALO JR. L. D. ET AL.,: "Trageting antigen into the phagocytic pathway in vivo induces protective tumour immunity" NATURE MEDICINE, XP002066618 see the whole document</p> <p>---</p>	1-47
A	<p>FU T.-M. ET AL.,: "Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization" J. VIROLOGY, vol. 71, no. 4, - April 1997 pages 27151-2721, XP002066619 cited in the application see the whole document</p> <p>-----</p>	1-47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00325

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 42,43,45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00325

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9505399	A	23-02-1995	US 5635602 A	03-06-1997
US 4587044	A	06-05-1986	NONE	

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

This Page Blank (uspto)